NEW CHARACTERISTICS FOR COLLAGEN-BASED SCAFFOLDS IN REGENERATIVE MEDICINE:

Modulating 3D structure and biomechanical properties

Luuk Versteegden

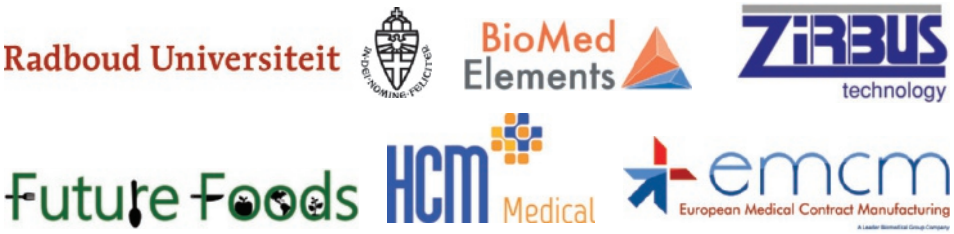
NEW CHARACTERISTICS FOR COLLAGEN-BASED SCAFFOLDS IN REGENERATIVE MEDICINE:

Modulating 3D structure and biomechanical properties

Luuk Versteegden

The work presented in this thesis was performed within the Radboud Institute for Molecular Life Sciences at the Department of Biochemistry, Radboud university medical center, Nijmegen, The Netherlands, and was funded by PIDON, a combined subsidiary program of the Dutch Ministry of Economic Affairs and the states of Gelderland and Overijssel (PID101020).

The printing and lay-out of this thesis was financially supported by the Radboud University, EMCM B.V., Zirbus Technology B.V., HCM Medical B.V., BioMed Elements B.V. and Future Foods B.V.



ISBN
978-94-028-1002-8

Cover art
Lara Leijtens, persoonlijkproefschrift.nl

Design/lay-out
Lara Leijtens, persoonlijkproefschrift.nl

Print
Ipskamp Printing, Enschede

NEW CHARACTERISTICS FOR COLLAGEN-BASED SCAFFOLDS IN REGENERATIVE MEDICINE:

Modulating 3D structure and biomechanical properties

Proefschrift

ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de rector magnificus prof. dr. J.H.J.M. van Krieken,
volgens besluit van het college van decanen
in het openbaar te verdedigen op donderdag 7 juni 2018
om 12:30 uur precies

door

Luuk Roland Maria Versteegden
geboren op 17 december 1988
te Lieshout

Promotor
Prof. dr. R.E. Brock

Copromotoren
Dr. T.H. van Kuppevelt
Dr. ir. W.F. Daamen

Manuscriptcommissie
Prof. dr. D.J.O. Ulrich (voorzitter)
Prof. dr. G.J.V.M. van Osch (Erasmus MC, Rotterdam)
Dr. ing. M.J.G. Wilmer

Paranimfen
Henk Hoogenkamp
Dirk Versteegden

Contents

Aims and outline of this thesis

The general aim of the research presented in this thesis was to contribute to the translation of collagen-based scaffolds from bench-to-bedside. The first part reviews recent literature and addresses novel insights and points to consider relevant in the process of clinical translation of tissue engineered biomaterials. **Chapter 1** is an extensive overview of currently available biomaterials and processing methods for the preparation of implants in the field of tissue engineering with a strong focus on extracellular matrix-derived materials. This chapter also addresses opportunities and critical issues for reseachers to optimize collagen scaffolds and to speed up clinical translation. In **Chapter 2**, a systematic overview is provided on all available literature on tissue engineering of the urethra including a meta-analysis in order to obtain an unbiased and comprehensive overview of the field, ultimately to facilate translation of pre-clinical research to the patient. The review reveals trends with regard to the use of different biomaterials and the use of (stem) cells in urethral implants in both pre-clinical and clinical studies. It also suggests recommendations on how to improve clinical translation.

In the second part of this thesis, new techniques are described to modify the 3D-structure and biomechanical properties of collagen scaffolds, as these are often not in compliance with the tissue to be regenerated. **Chapter 3** describes a novel technique to induce elastic-like characteristics to tubular scaffold consisting of solely type I collagen fibrils using straightforward compression, corrugation and chemical crosslinking. In **Chapter 4**, the development of an elastic tubular scaffold is continued by applying the processing technique of Chapter 3 to prepare a tubular scaffold with radial elasticity. **Chapter 5** presents an innovative collagen implant with the ability to self-expand upon exposure to water prepared by applying a method opposite to the one in Chapter 4. In **Chapter 6**, a new method is described to create stiff and rigid collagen scaffolds, which is based on treatment with concentrated salt solutions. This method may be useful for the preparation of collagen-based biomaterials with a defined stiffness. Finally, **Chapter 7** provides a summary of this thesis as well as future perspectives of the results described in this thesis.

EXTRACELLULAR MATRIX DERIVED BIOMATERIALS:

Molecularly defined ingredients and processing techniques

Henk R. Hoogenkamp
Luuk R. Versteegden
Toin H. van Kuppevelt
Willeke F. Daamen

Adapted from: Handbook of Intelligent Scaffolds for Tissue Engineering and Regenerative Medicine, Chapter 28, 2nd ed., edited by Gilson Khang, Pan Stanford Publishing, p 793-874

1. Introduction

Regenerative medicine (RM) is a new rapidly growing discipline in medical science. The need for RM partially originates from the chronic shortage of autologous or allogeneic tissues and organs for transplantation in cases of e.g., trauma, organ failure, tumors and general necrosis. The drawback of traditional allo- and xenogeneic organ or tissue transplantations is that, due to the genetic variation of species and individuals, it is subject to immunological compatibility complications which increases the severity of the organ shortage [1]. RM is based on combining knowledge gathered from the fields of molecular life sciences, biomedical engineering, (bio)materials science, reconstructive surgery and transplantation biology, to develop biomedical devices and treatments which aid in the repair or replacement of damaged tissues and organs [2]. More specifically, these biomedical devices or treatments comprise the supplementation to the damaged site of vital cells, extracellular matrix, biomolecules or a combination thereof. This central dogma has led to one important strategy, namely, the development of materials that mimic the extracellular matrix (ECM).

The ECM has many functions e.g., providing structural support for cells to reside, determining the mechanical properties of the tissues, providing mechanical signals to allow for cellular response and acting as a growth factor reservoir. Major ECM components in tissues and organs are collagens, elastin, laminins, fibronectins, proteoglycans and glycosaminoglycans [3]. The strategy is based on stimulating the body's regenerative capacity by implanting natural or synthetic ECM materials into a defect with or without cells. The ECM essentially acts as a template in the early stage of regeneration for cells to adhere or migrate to, proliferate and differentiate. Ideally, this hinders the formation of scar tissue and subsequently stimulates formation of new functional tissues, thus regenerating the organ's function [4]. These extracellular matrices, also referred to as biomatrices or scaffolds, can be classified into two groups: the decellularized tissues [5] or molecularly defined constructs comprised of natural and/or synthetic biomaterials [6].

This chapter aims to provide a fundamental and tutorial overview of the ECM and its components and highlights its essential role in RM of (soft) tissues. Subsequently, the current techniques used to process the ECM components into scaffolds for different applications are summarized and comprehensively reviewed.

2. The extracellular matrix

2.1. Role of the Extracellular Matrix (ECM)

In multicellular organisms the same set of biological rules generally apply. Cells can specialize in certain tasks, which in turn allow them to be more efficient. Due to the evolutionary process, cells form tissues in larger organisms, which in turn form organs that carry out a special function [7]. The cells are genetically identical, however, their gene expression depends on their function. The expression of specific genes leads them to produce biomolecules like proteins, carbohydrates, fats and metabolites. The cells are surrounded and subsequently held together by the ECM, which is produced by the cells themselves. The ECM is a dynamic and multifarious network that surrounds cells, providing structural and mechanical support in all tissues, mediating diverse biological processes that are crucial for supporting tissue formation and function, and playing an important role in wound healing. Cells adhere to the ECM via receptors (e.g. integrins) to maintain tissue architecture *in vivo*. ECMs can therefore regulate cellular functions by directly activating intracellular signaling pathways [8]. Depending on the function of the organ, the composition of cells and ECM differ tremendously. The composition of the ECM is specialized in different tissues in order to employ tissue specific cellular functions. For example, organs that have loadbearing functions like bone, cartilage, tendons and ligaments often have ECM's which are either rich in collagen, minerals or a combination thereof [9]. Organs with functions that require repetitive motions often contain collagen and elastin as the main structural component of the ECM like in the skin, lung and diaphragm [10]. In different tissues different ECM structures can be found, where for example the epithelial and dermal layer have dissimilar compositions. The complexity increases when taking into account that the composition of ECM constantly changes along with developmental stages of an organism [11] and also through pathological conditions, like fibrosis after trauma [12].

2.2. ECM constituents

The ECM mainly consists of collagens, elastin, proteoglycans, laminins, fibronectins and glycosaminoglycans. Collagen provides tissues with essential tensile strength, enabling resistance to plastic deformation and rupture [13]. Type I collagen is the most abundant collagen and is present as a fibrillar protein, often accompanied by other collagen types like II, III, IV and V [14]. Elastin on the other hand, provides tissues the properties of extensibility and reversible recoil, enabling tissues to withstand repetitive mechanical stress [15]. The ECM is filled with a viscous interstitial fluid rich in proteoglycans (PGs) and other

glycoproteins, containing a complex mixture of proteases, growth factors, cytokines and other effector molecules. Fibronectins can be found as a structural insoluble glycoprotein or soluble plasma protein, and contains binding domains that can immobilize other ECM components. Laminins are large trimeric glycoproteins that can be found in basement membranes. Laminins also have the ability to bind ECM components and can interact with cells with specific domains. Depending on the organ/tissue function different collagens can be found with different combinations of elastin, laminins, fibronectins and PGs. PGs consist of a core protein with a varying amount of glycosaminoglycans (GAGs) chains attached to them. GAGs are long, linear and strongly negatively charged polysaccharides, containing repeating disaccharides. GAGs are an important part of the ECM and govern countless functions from cell growth and differentiation to wound healing and cell protection. These components will be further elaborated next.

3. Molecularly defined biomaterials

One of the two fundamentally different strategies to design the scaffolds follows the "bottom-up" philosophy, making use of molecularly defined biomaterials [3, 16]. This section will discuss molecularly defined biomaterials produced from naturally occurring ECM components. In contrast to the decellularization strategy, components of molecularly defined scaffolds are individually purified and can subsequently be used in combination with a variety of processing methods. Substituting the use of cadaver tissue can improve standardization, quality and safety issues that inherently accompany decellularization strategies (see section 4.6). Advantages of molecularly defined materials are related to increased versatility, controllability, reproducibility and safety of the products. The potential and subsequent advantages of using defined biomaterials have been demonstrated in numerous studies [16-20]. Typically, depending on the type of molecularly defined biomaterials, they can be processed into different types of scaffolds (discussed in section 4). Biological and mechanical prerequisites of the defined biomaterials in question are **(i)** to support and deliver cells, **(ii)** to induce and differentiate tissue growth, **(iii)** to function as cell adhesion substrate, and **(iv)** to stimulate cellular response. Other important materials properties include **(v)** formation of a wound-healing barrier, **(vi)** biocompatibility and controlled (non)-biodegradability, **(vii)** relative ease of processability and malleability into desired shapes, **(viii)** mechanical strength and dimensional stability and **(ix)** sterilizability [21]. The used materials can either be of natural or synthetic origin. In this chapter only materials found in the ECM will be discussed.

3.1. Mammalian ECM-based Materials

Biomaterials isolated from natural sources have been widely used in the RM arena. Early research focused on proteins isolated from the human ECM and their roles in wound healing. From the primary amino acid sequence up to the tertiary/quaternary structure of extracellular matrix proteins, the degree of conservation is generally high throughout primates and ungulates. A high degree of homology in structure might theoretically reduce the chance of an unsolicited host immune response. Therefore,

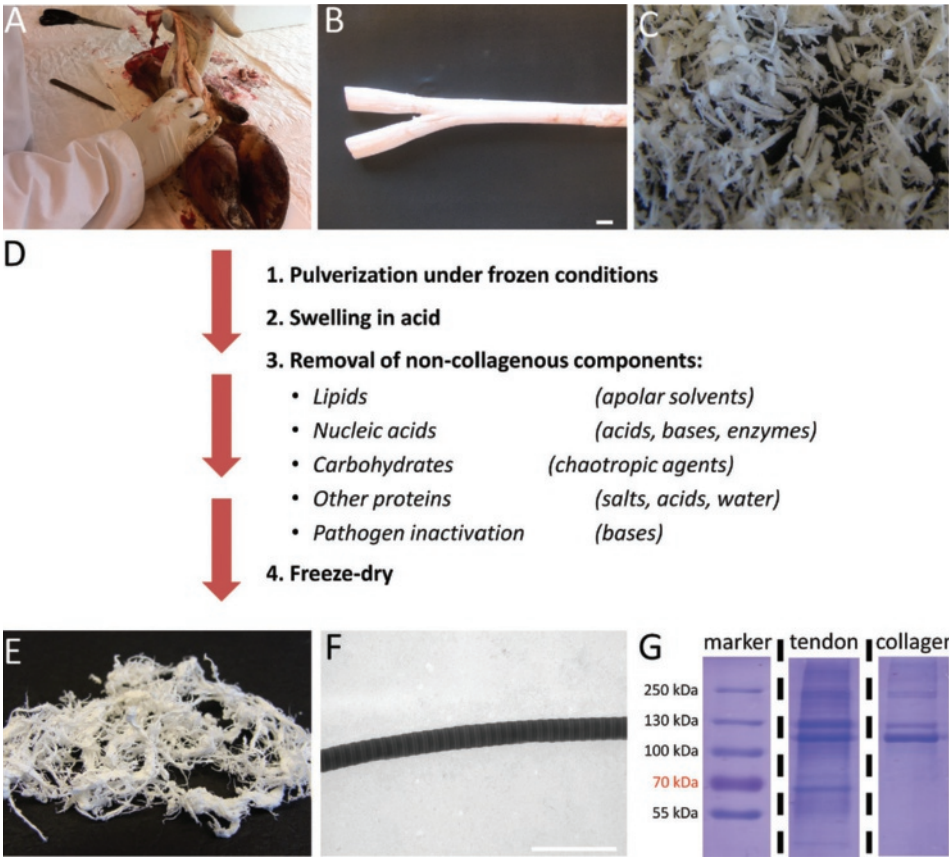


Figure 1: Isolation of collagen as a biomaterial. A) Achilles tendons are removed from bovine legs and B) subsequently freed from macroscopical impurities such as fatty membranes and blood, bar is 1 cm. C) The cleaned tendon is pulverized under frozen conditions followed by D) washing steps with acids, bases, chaotropic agents, salts and organic solvents to remove non-collagenous components. E) This procedure results in purified collagen fibrils as a basic material. Several quality assurance steps can be taken; F) transmission electron microscopy (TEM) to visualize the quarter staggered array structure of the collagen fibrils, bar is 500 nm and G) SDS-PAGE to assess protein impurities in the final product. The characteristic collagen bands can be observed and the isolated collagen does not contain other proteins found in the raw material (tendon).

many attempts have been made to identify ubiquitous sources of the ECM specific component in question, in genetically related mammalian species. Next to ECM proteins, polysaccharides (e.g., GAGs) are also subject to substantial research interest due to their versatility and roles they play in the ECM. In general, the organs of interest are retrieved from a standardized source after which the surface area is increased (pulverization) to facilitate future processing steps. The material is then subjected to an array of carefully selected chemicals and enzymes to remove the fractions that are not of interest. After isolation, the ECM component is subjected to tests in order to determine its purity and (near) native state. This central scheme is found in most processes used to obtain ECM components (see figure 1). Proteins and GAGs are the main component of the human ECM. However, many other natural materials have been isolated from other non-mammalian sources like for example from plants or crustaceans. Even though these isolated materials are not native to the human physiology, some are biocompatible and can mimic certain functions of the natural ECM. In this section an overview will be given of the different available natural materials currently being used in RM research.

3.1.1. Collagen

Collagen is the most abundant protein in man and most other vertebrates. It is a major structural polymer which can be found throughout the body providing structural integrity and rigidity in tissues like tendons, cartilage and skin [22]. Along with calcium, collagen is important for strength and structure in bones and teeth. So far more than 28 genetically distinct types of collagen have been found, of which type I collagen is most abundant [23]. On a cellular level, type I collagen mainly acts as a structural component but it also mediates biological functions like cell binding, migration, growth and chemotaxis [24]. Type II collagen is predominantly found in cartilage whereas type III collagen can be found in more elastic tissues like the skin. Type IV collagen is a universal component of the basement membrane, a thin sheet of specialized ECM upon which a large number of cell types (epithelium, endothelium, muscle cells) rest and which plays an essential role in cell adhesion. Collagen-based biomaterials have been widely used for tissue engineering applications for a number of reasons, including biocompatibility, biodegradability, low immunogenicity and low antigenicity [25].

3.1.1.1. Collagen Biosynthesis and Structure

Figure 2 shows a schematic representation of the collagen biosynthesis. The consensus sequence $(\text{glycine-X-Y})_n$ gives rise to α-chains, the peptide subunit, of the fibril-

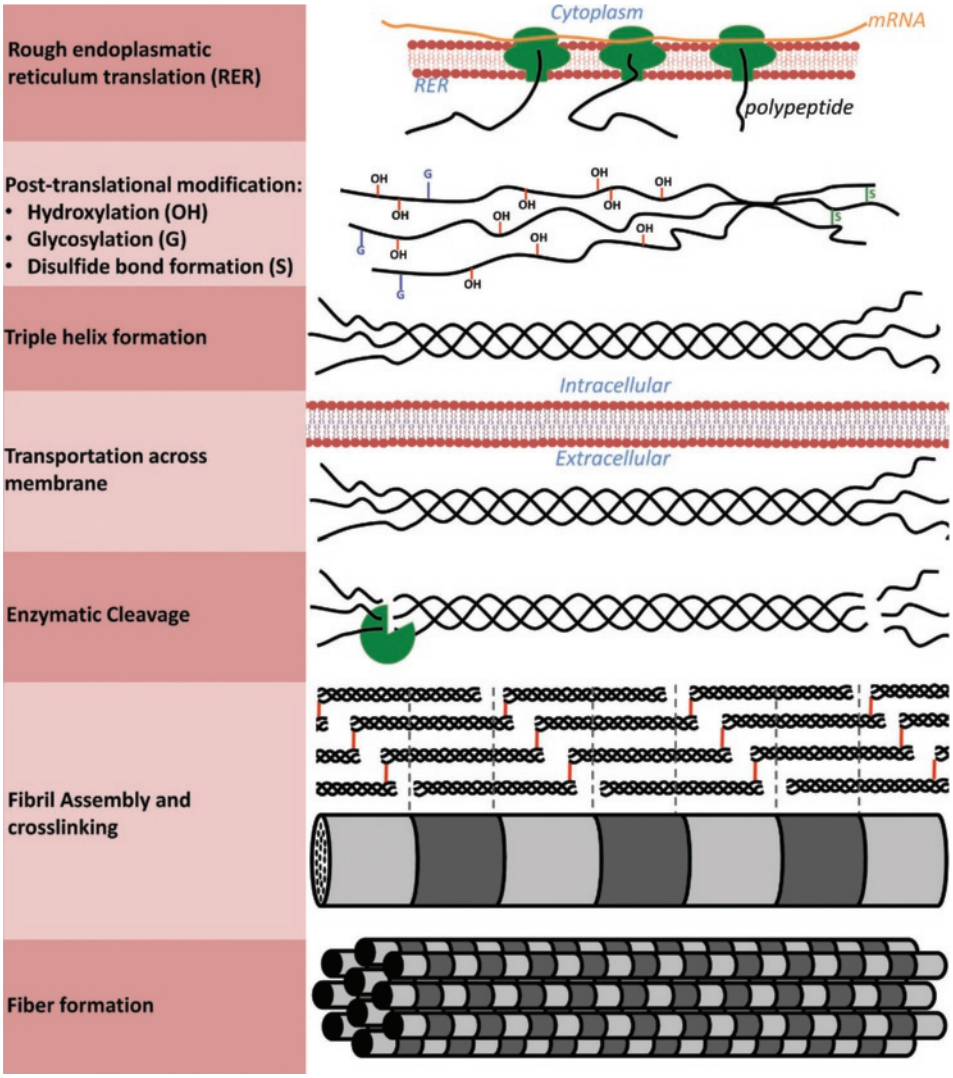


Figure 2: Cartoonized scheme of the synthesis and structure of collagen. As with all proteins, collagen mRNA is translated by ribosomes into individual polypeptides (pre-procollagen subunit) of which 3 assemble (procollagen) whilst undergoing post translational modification such as; hydroxylation, glycosylation and disulfide bond formation. The triple helix is formed and subsequently transported outside the cell where the N- and C-propeptides are cleaved off. The cleaved monomeric collagen (tropocollagen) is then involved in the self-assembly into collagen fibers with the characteristic quarter staggered array or banding pattern. The monomeric collagen overlaps approximately ¼ of each molecule, creating a dense and less-dense area, also referred to as the overlap D-periodicity. The monomers are subsequently fixed by crosslinks. In most cases the fibrils bundle and form larger collagen fibers.

forming collagen helix (types I, II, III, V, and XI) [26]. The X and Y can be any amino acid however; the positions are frequently occupied by proline and hydroxyproline, respectively. The hydroxyproline and proline provide rigidity due to their unusual ring-like structure in the peptide backbone and force the peptide chain into a left-handed helix. Three identical α-chains (homotrimers in the case of e.g. collagen types II and III) or two or more different α-chains (heterotrimers in the case of e.g. collagen types I and IV) are twisted together to form a right-handed triple helix. This triple helix is also known as tropocollagen and is, like DNA, stabilized by hydrogen bonds [26]. The smallest amino acid (glycine) allows tight packing of the three α-chains and provides flexibility to the peptide backbone. Tropocollagen is the basic molecular unit of "collagen" with a general molecular weight of about 300 kDa, length of 280 nm and width of 1.5 nm [27]. At the ends of the collagen molecule, there are areas characterized by a lack of hydroxyproline and proline and subsequent lack of triple helical structure. These 'frayed' ends of the collagen molecule are also referred to as the telopeptide regions (at both the N- and C-terminal ends). The telopeptide region is rich in lysine and hydroxylysine residues and has four main functions, namely: i) the stabilization of the molecule via intramolecular crosslinking, ii) the formation and stabilization of collagen fibrils via intermolecular crosslinking, iii) the crosslinking of points with other extracellular matrix proteins and **iv)** the formation of "sticky ends" by disulfide bonds to aid self-assembly [28]. The tropocollagen molecules assemble in a quarter-staggered fibril array, so that each molecule overlaps approximately one-fourth with its neighboring molecule (67 nm). This process repeats itself until a fibril of a certain dimension is formed. This phenomenon can be demonstrated under the transmission electron microscope, where a characteristic banding pattern of alternating light and dark striation can be seen. This characteristic banding pattern has a periodicity of 65-70 nm (depending on the tissue) and is also referred to as the D-period [29]. The overlapping arrangement of the collagen molecules are stabilized by the formation of intermolecular covalent crosslinks. Most crosslinks are formed between N- and C-telopeptide ends and certain residues within the triple helical part of other neighboring molecules. During fibril formation non-covalent and reducible crosslinks are also formed between the molecules, based on either lysine aldehydes (aldimine bonds), hydroxylysine aldehydes (ketoimine bonds) or a combination thereof [30]. During maturation of the tissue, these crosslinks are converted into non-reducible bonds [31]. The N-telopeptide is believed to play an important role in regulation of fiber thickness, although this process is not yet fully understood [32].

Other insights in collagen synthesis have led the community to believe that the incorporation of other types of collagen (i.e. type V, IX, XII or XIV) or other non-collagenous proteins during the fibril formation controls fibril thickness [33]. The fibril diameter and the distribution of different fibril dimensions is an important determinant of the mechanical properties of the tissue in question. Thick fibrils exhibit great tensile strength but are susceptible to creep (plastic-deformation). It has been suggested that thinner fibrils may be more efficient at inhibiting creep due to the larger surface area in contact with the rest of the matrix over which the shear stress dissipates [34]. Every tissue and inherent function has thus a specific distribution of collagen fibers with varying diameters [35]. Collagen fibrils can bundle to form even thicker collagen fibers [36]. These bundles of fibrils (fibers) are not connected to each other in a similar manner as the molecules within the fibrils. Instead the structure can be viewed as a highly interwoven set of fibrils/fibers [37].

3.1.1.2. Collagen Applications

Collagen is regarded as one of the most versatile biomaterials in both hard and soft tissue engineering. Along with the mechanical and biological role in the ECM in both homeostasis and wound healing, the ubiquitous occurrence of collagen throughout the human body makes it an ideal candidate for use as a biomaterial. Due to its natural role in the ECM, type I collagen possesses worthy mechanical characteristics for a protein [38]. Fibrillar collagen is considered as a robust protein, which can endure relatively high temperatures and is, to a certain extent, resistant to corrosive processing chemicals like salts, acids, bases and detergents [39]. Its general robustness makes collagen compatible with many processing techniques. The properties of collagen can be influenced using chemicals, enzymes and physical processes like drying or heating [40]. For example, mechanical and biochemical characteristics can be modified by crosslinking (see section 4.7) or functionalized by covalent addition of glycosaminoglycans [41]. One of collagen's unique properties is the reversible process of pH driven precipitation/fibrillation [42]. Collagen present at pH values where the net charge of the collagen molecules is either maximally negative or positive causes the molecules to repel each other and subsequently increase water uptake capacity. This phenomenon is referred to as 'swelling' and can be utilized in modification of its properties (see figure 3) [43]. Swollen collagen can be subjected to homogenization techniques to create a viscous fibrillar paste, which can then be formed into different scaffold types (see section 4).

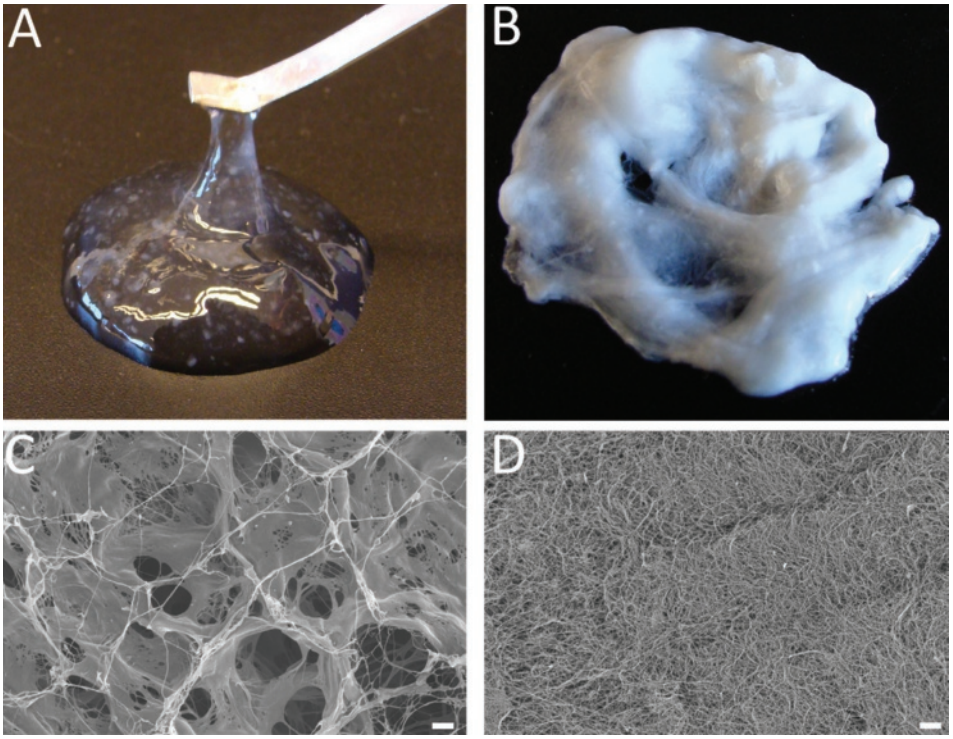


Figure 3: Reversible swelling and precipitation of collagen fibers. A) 0.5% w/v swollen collagen fibril suspension in 0.25 M acetic acid at pH 3.0. B) Collagen that was precipitated by neutralizing the suspension to pH 7.4. SEM images show C) the acid swollen collagen and D) the precipitated collagen after freezing and freeze-drying displaying either a porous or fibrous structure, bars are 10 µm.

Fibrillar collagen has been studied in numerous RM applications where biodegradability, porosity and mechanical strength were required [17, 44, 45]. Moreover, fibrillar collagen at an acidic pH can subsequently be digested to yield a truncated form of molecular collagen where the telo-peptide ends are cleaved using pepsin, resulting in atelocollagen [46]. This monomeric collagen resembles the native form of molecular collagen (acid soluble collagen) and both exhibit characteristics differing from fibrillar collagen. Molecular or monomeric collagen is completely soluble in water or acid and subsequently forms a viscous solution. Under physiological conditions (pH, solutes, temperature, etc.), fibrillogenesis can occur where *de novo* collagen fibrils are formed, in turn forming a hydrogel. Collagen hydrogels, or hydrogels in general, have a wide array of applications which are listed in section 4.2. Although successful in various applications, scaffolds based on monomeric collagen suffer from non-physiological small fibril dimension and poor mechanical strength [47]. Depending on the ultimate

goal, the previously discussed items should be taken into consideration when working with collagen as a biomaterial.

3.1.2. Gelatin

Gelatin, a soluble protein derived from the unfolding or partial hydrolysis of collagen, is highly biocompatible and biodegradable in a physiological environment [48]. The conversion of collagen into gelatin, or also referred to as a helix-to-coil transition, has been extensively studied in various settings [49]. Basically, gelatin is a mixture of collagen α-chains (single peptide chain), β-chains (two α-chains) and γ-chains (three α-chains) or any intermediates thereof. Depending on the state of the collagenous material, be it in fibers, fibrils or single molecules, a combination of acids, bases and heat treatment can be used to break the covalent and non-covalent bonds in order to destabilize the collagen triple helix [50]. The treatment method subsequently affects the final properties and generally results in either of two types of gelatin, commercially known as type-A gelatin (acid treated, isoelectric point between pH 8 and 9) and type-B gelatin (base treated, isoelectric point between pH 4 and 5) [51]. Gelatin has a unique combination of properties for a protein, which make it a versatile biomaterial, i.e. from thermo-reversible gels to porous materials, films, capsules and electrospun fibers [52]. Gelatin is generally regarded as having low-antigenicity and the metabolic products thereof are harmless since collagen is normally degraded by proteolytic hydrolysis in the body [53]. Due to the aforementioned reasons, gelatin has been frequently used in drug carrier and delivery systems [54], as a plasma expander [55], wound dressing [56], medical device coating [57] and as a basis for RM applications in sponges, films and hydro/cryogels [58]. Gelatin has many applications within RM and is often combined with other carbohydrate-based biomaterials (e.g., chitosan, alginate and agarose) and an array of processing techniques (electrospinning, coacervation and crosslinking) [59].

3.1.3. Elastin

Elastin is a vital ECM protein that provides elasticity to tissues and organs [60]. Elastin comprises up to 70% of the dry weight in elastic ligaments, 50% in large arteries, 30% in lung and 2-4% in skin, where the fibers are present as rope-like structures [61]. Its primary role is to allow tissues to undergo repetitive deformation and subsequently facilitate the return to its original dimensions when the force is released [62]. It is generally accepted that the spontaneous recoil of stretched elastin is entropic in origin, where in its extended mode all the energy is taken up by the polypeptide backbone and is recovered upon relaxation [63]. Water-soluble tropoelastin monomers are

alternatively spliced polypeptides with sizes ranging from 60-70 kDa. The monomers have characteristic alternating hydrophobic and crosslinking domains. When the tropoelastin molecules are transported outside the cell, the hydrophobic domains regulate the association with other tropoelastin molecules and the lysine and alanine-rich hydrophilic domains ensure the presence of free amine groups for subsequent crosslinking by lysyl oxidase to form insoluble elastin [64]. Unique to elastin is the formation of desmosine and isodesmosine, where four (modified) lysine residues from two tropoelastin molecules react with each other to form tetrafunctional crosslinks [65]. Elastin molecules are abundantly crosslinked to stabilize the structure and induce the elastic coil properties. Elastin has also been identified as a signaling molecule, governing cellular responses like chemotaxis, proliferation and differentiation [66]. Elastin can interact directly with cells through different cell-surface receptors like the elastin/laminin receptor which has been implicated in elastin assembly, cellular processes and interaction with other ECM components like GAGs, fibrillin and laminin [67].

Due to its versatility, elastin has been subject of investigation for use in RM applications. However, due the extensive crosslinking and subsequent insolubility, elastin is very difficult to manipulate limiting its use as a biomaterial. Elastin can be solubilized by enzymatic or chemical hydrolysis to make it more suitable for use in scaffolding. However, along with the solubilization, the elastin partially loses its mechanical properties. The soluble elastin can still functionalize biomaterials. Soluble elastin can be coated on natural or synthetic polymers to modify the surface hydrophobicity [68], decrease thrombogenicity [69], increase angiogenesis [70] and improve the cell adhesion properties [71]. Three-dimensional scaffolds have been made from solubilized elastin as a hydrogel-based material[72] or as an electrospun sheet or tube [73]. Incorporation of both soluble and insoluble elastin in scaffolds has been investigated for use in dermal [74] and vascular applications [75]. For more elaborate description of the applications of elastin-derived biomaterials the readers are referred to excellent reviews by Almine *et al.* [76], Daamen *et al.* [77] and Sivaraman *et al.* [78].

3.1.4. Adhesive glycoproteins

Cells adhere to the ECM through interaction between cell surface proteins and adhesive ECM glycoproteins, including fibronectins, laminins, fibrinogen, entactins, vitronectin, thrombospondins, tenascins, nephronectin, and others [79]. Most adhesive glycoproteins play an important role in the cell attachment, movement and differentiation [80]. Cell surface proteins (mostly integrins) recognize certain

domains within the adhesive glycoproteins for example the tripeptide sequence Arg-Gly-Asp (RGD). This sequence is crucial for the interaction of the adhesive glycoprotein with its respective cell surface receptor [81]. Next to cell surface proteins, adhesive glycoproteins possesses multiple binding domains capable of binding other ECM components like collagen, elastin, proteoglycans, GAGs and other adhesive glycoproteins [82]. This section will shortly describe the adhesive glycoproteins laminin, fibronectin and fibrinogen, which are of interest to RM applications. Other adhesive glycoproteins have received much less attention as of yet due to their relatively unknown role in organogenesis. These include vitronectins, thrombospondins, tenascins, entactins and nephronectin, which all have their specific effects on cell and protein binding, ECM remodeling and wound healing [83]. Moreover, glycoproteins can be involved in tissue transplant rejection and this should be taken into consideration in designing RM applications [84]. Taking specific peptide sequences responsible for a certain function of the respective proteins, e.g., adhesion, migration or proliferation, can circumvent immunogenic responses against these adhesive glycoproteins [85, 86]. Next to the previously mentioned RGD, the YIGSR and IKVAV sequences are also used for cellular adhesion to surfaces [87, 88]. Also, short sequences from collagen [89] and proteoglycans [90] are being used in similar manners to controllably elicit desired cellular responses. Straightforward peptide chemistry could be used to synthesize functional short polypeptides. Hence, expensive preparation methods of full-length proteins such as purification from an animal sources or recombinant DNA techniques could be avoided.

3.1.4.1. Laminin

Laminins are considered to be one of the major adhesive glycoproteins and are a main component of the basement membrane. Laminins are large heterotrimeric glycoproteins consisting of α, β and γ chains. Due to alternative mRNA splicing, different chains can be synthesized respectively. Around fourteen chain combinations have been described *in vivo* and each molecule is named according to their chain composition [91]. It is generally believed that the cross-like structure of laminin facilitates both cell adhesion as well as laminin sheet or basement membrane formation. Additionally, laminin has a high affinity for type IV collagen and heparan sulfate, which are also major components of the basement membrane [92]. Laminins generally play important roles in cell adhesion, migration, proliferation, neurite outgrowth, and angiogenesis [93]. Due to its high affinity for ECM components and cell surface proteins, laminin is widely used in the coating of cell culture surfaces or scaffolds [94].

3.1.4.2. Fibronectin

Fibronectin shows functional similarities with laminin in its roles in the ECM. Fibronectin is a protein dimer produced from a single gene and consisting of nearly identical monomers that are linked by disulfide bridges. Each subunit contains three types of repeating modules (FN1, FN2 and FN3) linked by short connector modules. Due to alternative splicing, which is regulated by cell type and stage of development, differences in these subunits are induced and have resulted in over 20 types of human fibronectin variants [95]. Early on in ECM formation, fibronectin is assembled into a fibrillar network on the cell surface providing deposition points for fibronectin-interacting ECM proteins like collagens, fibrin and heparan sulfate proteoglycans [96].

3.1.4.3. Fibrinogen

The last major adhesive glycoprotein is fibrinogen. Fibrinogen is a large (349 kDa), soluble and complex protein consisting of three different subunits (Aα, Bβ and γ) varying in amino acid length (610, 461 and 411, respectively) linked by disulfide bonds [97]. Fibrinopeptides on the soluble fibrinogen can be cleaved by thrombin to convert fibrinogen into insoluble fibrin. Fibrin molecules assemble to form a protofibril, which subsequently aggregate into larger fibers. A 3D network, or a fibrin clot, is formed which is capable of catching blood platelets to promote aggregation. This is essential for forming a hemostatic barrier and the newly formed network offers temporary scaffolding for cells involved in wound healing. Transglutaminase (factor XIIIA) crosslinks the glutamine and the lysine residues in the fibrin clot which temporarily stabilizes the network against chemical, mechanical and proteolytic degradation [98]. Fibrin, or forming fibrin clots, also has affinity for some plasma proteins such as fibronectin and albumin, which have influence on properties like fibril thickness and density [99]. Other (glyco)proteins like thrombospondin, von Willebrand factor and fibulin bound to the fibrin clot increase the affinity for blood platelets. Moreover, growth factors like FGF-2 and VEGF are also bound to stimulate swift remodeling of the damaged site. It is noteworthy, that because of the presence of other proteins, clots formed *in vivo* have different properties compared with clots formed with purified components.

3.1.4.4. Application of Adhesive Glycoproteins

Laminin, fibronectin and fibrinogen have received much interest from the RM community due to their innate role in wound healing and ECM formation [100]. Also their role in cell-cell and cell-ECM adhesion during both tissue growth and tissue

homeostasis makes them useful components in RM. Being a minor ECM component and potentially immunogenic, laminin is less suitable for use as a structural scaffolding but more so as a coating material. The most frequent use of laminin can be traced to *in vitro* culture of various cell lines and primary cells [101]. In RM of the central nervous system, both synthetic [102] and natural materials [103] have been coated with laminin to improve cellular compatibility [104]. Laminin has also been incorporated in hydrogels to improve cell adhesion in central nervous system [105], intervertebral disc [106] and pancreas [107] applications. As fibronectin also increases cellular adhesion, it has been used in similar applications as laminin [108]. Some effort has gone out to link fibronectin to biomaterials via laser patterning [109], chemical conjugation [110] and genipin crosslinking [111]. Fibronectin has also been used frequently in combination with fibrinogen as a scaffolding material [112]. Perhaps the most versatile of the adhesive glycoproteins is fibrinogen and/or fibrin. Due to its innate clotting ability after cleavage by thrombin, it is highly suitable for use as a surgical adjuvant in hemostatic, sealing or adhesive applications [113]. The use of fibrin in modern medicine has therefore received most attention and has been applied in the clinic extensively [114]. The versatility of fibrinogen/fibrin has been demonstrated by its compatibility of a wide range of scaffolding techniques including, but not limited to, hydrogel formation [115], electrospinning [116], coating [117], particulate leaching [118] and others. The use of fibrin has also been researched for nearly every RM application including adipose [119], bone [120], cardiac [121], cartilage [122], muscle [123], neural [124], ocular [125], respiratory [126], skin [127], tendon [128], ligament [129] and vascular tissue [130]. For further reading, several reviews of fibrin in RM are available [131, 132].

3.1.5. Keratin

There are countless other proteins inside and outside the cell that were not discussed in the previous text. As proteins can be classified by their shape as for example fibrous or globular, the structural proteins are usually regarded as fibrous [133]. Despite being the main component of horse tail-hair, an early naturally occurring suturing material [134, 135], the fibrous protein keratin, has enjoyed only marginal research for RM applications. Keratins are epithelial-specific family members of the superfamily of intermediate filament structural proteins that are of paramount importance in the outer layer of the skin, hair, nails and horns. The keratin sub-family comprises of types I and II, of which respectively, 28 and 26 genetic variations are known. Keratins fulfill two main fundamental roles in epithelial cells where they provide structural support and participate in the regulation of metabolic processes like proliferation, migration and

apoptosis [136]. The soluble keratin monomers assemble into bundles and in turn form insoluble intermediate filaments [137]. The exact process of assembly remains unclear, but for further information the readers are referred to a review by Kölsch *et al*. [138].

Keratin has been considered as a biomaterial by several research groups due to several key features. For example, structures containing large amounts of the assembled keratin filaments are generally thermostable and possess great mechanical strength [139]. Evidence also suggest that keratin is biocompatible and does not induce obvious toxic effects both *in vitro* and *in vivo* [140]. Moreover, keratin-based biomaterials have an advantage in that they do not degrade by the same mechanisms as other proteins because of the lack of keratinases in mammals. The breakdown of keratin-based biomaterials is regulated by phagocytosis and ubiquitin systems [141]. This subsequently allows keratin-based scaffolds to persist longer than other protein-based scaffolds [142]. Keratin-based biomaterials in the form of sponges and films have been produced from wool and human hair for various biomedical applications such as wound dressings a neural tissue engineering applications [143, 144]. Keratin can also be processed into a hydrogel and has shown to support nerve cell migration and proliferation [145]. Keratin-based hydrogels in combination with commercially available nerve conduits have been used in small mammal studies to replace peripheral nerve segments and proved to be equivalent or more effective autografts [142, 146]. The use of keratin for its great mechanical strength has also attracted interest from the hard tissue arena, where it has also shown to a versatile biomaterial [147, 148].

3.1.6. Proteoglycans and Glycosaminoglycans

Proteoglycans (PGs) are a class of proteins that harbor one to many glycosaminoglycans (GAGs). A proteoglycan consists of a core protein to which GAGs are covalently attached. PGs influence and help regulate ECM assembly and collagen fibril formation [149]. This is important during inflammation, tissue repair and remodeling. Moreover, PGs play an important role in of a variety of tissues. PGs and GAGs contribute to the mechanical resilience of tissues by maintaining optimal visco-elastic characteristics, compressive stiffness and tissue homeostasis by sequestering water [150]. A number of other functions include regulation of coagulation, lipoprotein clearance in the liver, control of growth factor binding, and signaling. As in adhesive glycoproteins, PGs participate in both cell-cell and cell-matrix interactions. The attached GAGs largely determine the specific properties of a PG. GAGs are long, linear and strongly negatively charged polysaccharides, characterized by up to quantity hundred or so

repeating disaccharides. GAGs can be either sulfated or non-sulfated which also largely determines the overall PG charge.

Sulfated GAGs are usually classified into five types: heparan sulfate (HS) [151], heparin [152], chondroitin sulfate (CS) [153], dermatan sulfate (DS) [153] and keratan sulfate (KS) [154]. Hyaluronan (HA) is the only non-sulfated GAG [155]. Important PGs include perlecan (basement membrane, HS and CS) [156]; aggrecan (cartilage, CS) [157]; versican (connective tissue, CS and DS) [158]; neurocan and brevican (CNS tissue, CS) [159, 160]; biglycan, fibromodulin and decorin (collagen rich tissues, CS and DS) [161, 162]; bikunin (plasma, CS) [163]; lumican, keratocan, glypicans and syndecans (epithelial tissues and cornea, HS and KS) [164, 165]. GAGs also sequester a wide range of growth factors, consequently acting as a reservoir. Changes in physiological conditions, like trauma or infection, can trigger protease activities that cause local release of such depots allowing the rapid and local growth factor-mediated activation of cellular functions [166].

The structural diversity of the PGs and its respective GAG composition renders each PG and GAG unique in its biological function. Because of the ubiquitous presence of GAGs in healthy tissue ECM and their diverse functions in tissue homeostasis, GAGs have gained interests from biomaterial researchers. In RM, GAGs are frequently used to supplement existing scaffolds, made from natural materials, synthetic polymers or a combination thereof [167].

3.1.6.1. Heparin and Heparan Sulfate

Heparin and heparan sulfate (HS) are highly sulfated GAGs where heparin is associated with the highest negative charge density of any known biological molecule [168]. Native heparin and HS are, like other GAGs, polymers with varying molecular weight (10-70 kDa and 7-20 kDa, respectively) [169]. They consist of disaccharide units of N-acetyl-D-glucosamine and D-glucuronic acid, of which the latter sugar can be epimerized into L-iduronic acid during subsequent variable modification of the polymer. Other modifications include N-deacytelation/N-sulfation, 6-O and 3-O sulfation of the glucosamine and 2-O sulfation of the uronic acid. Alternatively, the glucosamine can have a free amine group on the C2 position instead, likely due to uncoupling of the N-deacetylation and N-sulfation [170]. Together with 3-O sulfation, this is the least prevalent modification. Heparin and HS are closely related due to the composition of the disaccharide structure, however the main difference being that heparin has about a

two-fold increase in N- and O-sulfation and subsequently two times its overall negative charge [171]. Whereas heparin is almost uniformly highly sulfated, HS contains domains of low, moderate and high sulfation [172]. The exact function of heparin and HS in the human body is still being elucidated but current literature holds plenty of evidence suggesting that they are both involved in a wide array of functions. Heparin is mainly produced and stored by basophils and mast cells, whereas HS can mainly be found on the cell surface and in the surrounding ECM. Heparin is used as a pharmaceutical anticoagulant. Medical devices that come into direct contact with the blood circulation are often coated with heparin to prevent blood clotting. Within the RM theme, heparin is utilized for its ability to bind an array of growth factors. Growth factors with heparin binding domains can be attached to heparin immobilized on a scaffold surface [173]. The attachment of heparin and subsequently heparin-binding growth factors like FGF2 and VEGF has shown to increase the angiogenesis in subcutaneously implanted collagen scaffolds [41]. Additionally, the attachment of heparin to (non-)biodegradable materials can influence the tissue response where the negative charge can influence the water holding capacity or the overall hydrophobicity of a construct. For further reading regarding the exact differences between heparin and HS the readers are referred to other sources [174, 175].

3.1.6.2. Chondroitin Sulfate

Chondroitin sulfate consists of two alternating monosaccharides, D-glucuronic acid and sulfated N-acetyl-D-galactosamine. With a molecular weight ranging from 20-60 kDa, CS is the most prevalent GAG in the human body and is an important component of cartilage where it plays a role in resistance of compressing forces [176]. CS comprises a group of 8 CS subtypes, CS-A, C, D, E, F, H, K, and O, of which the type is dependent on the sulfation pattern of the monosaccharides. The sulfations at different positions of the monosaccharide confer different biological activity to the CS-containing PG [177]. For example, in cartilage CS containing PGs like aggrecan are tightly packed with the collagen structure where the charged sulfate groups of the CS generate electrostatic repulsion that provides resistance to compression. For further details regarding the different subtypes the reader is referred to an overview [178]. The main medical application of CS lies in nutraceutical application for the treatment of osteoarthritis. Along with glucosamine, CS has been thought to pose anti-inflammatory properties although the possible underlying mechanism is poorly understood [179]. Within RM, CS has been subject of research for use in scaffolds to improve the biomechanical properties [180]. However, CS is currently mostly limited to osteochondral and cartilage

applications [181]. Like heparin (and HS), CS is able to bind growth factors and is therefore also able to modulate the bioactivity of the material [182]. CS has also found its way into a commercially available biomaterial application for skin regeneration by Integra Life Sciences, where a bilayer wound matrix consisting of type I bovine collagen bound CS and silicone is applied as a wound dressing [183].

3.1.6.3. Dermatan Sulfate

Dermatan sulfate is composed of disaccharide units consisting of N-acetyl-D-galactosamine and glucuronic/iduronic acid. Like CS, DS is defined by the presence of N-acetyl-D-galactosamine, however, the presence of iduronic acid distinguishes it from CS. DS is the predominant GAG expressed in the skin but is also found in blood vessels, heart valves, tendons and lungs. Several processes involve DS as a co-factor or regulator of growth factors, cytokines, and chemokines [184]. Moreover, DS plays an important role in signaling in response to the coagulation cascade and cellular damage and subsequent regeneration of the fibrous ECM [185]. DS as a biomaterial or component of biomaterials have enjoyed little attention [16]. Several attempts have been made to study the *in vitro* effect of DS on chondrocyte, fibroblast and keratinocyte function [186]. It is possible that difficulties in procurement of large quantities have hampered widespread study of DS in TE/RM applications. For further reading regarding the function of DS, an extensive overview is given by Trowbridge *et al*. [187].

3.1.6.4. Keratan Sulfate

Keratan sulfate (KS) is relatively the simplest of the sulfated GAGs, consisting of repeating disaccharides composed of galactose and N-acetyl-D-glucosamine with sulfation at the 6-O positions. The size of KS is highly variable and can range from 2 to 24 kDa [188]. KS is the major GAG in the cornea and can also be found in scar tissue of the central nervous system and in connective tissues like cartilage [189, 190]. During the formation of the cornea, KS interacts with collagen to assure the characteristic highly organized collagen fibril structure, which renders it transparent [191]. Like other GAGs, KS has water-binding capabilities and thus plays an important role in the maintenance of the transparency and general homeostasis of the cornea. The water binding in combination with fibrous proteins enables the tissue to endure mechanical wear and tear from compression and abrasion. KS has also been linked to the repair of corneal tissue where it is believed to paradoxically act as a cell de-adhesion molecule but also to facilitate the motility and attachment of the corneal epi- and endothelial cells [192, 193]. The application of KS in RM research has to date been limited to use as

coatings in cell culture to investigate cell motility [193]. However, synthetic analogues to proteoglycans (peptidoglycans) have been used more frequently in scaffold design [194]. For further reading please see literature on KS and the physical and biological aspects [195, 196].

3.1.6.5. Hyaluronic Acid

Hyaluronic acid, also referred to as hyaluronan or hyaluronate, is an anionic non-sulfated GAG and is composed of repeating disaccharide units containing D-glucuronic acid and N-acetyl-D-glucosamine. HA is a major constituent of synovial fluid but is also found in connective, neural and epithelial tissues. HA is unique from other GAGs since it is non-sulfated, not attached to a core protein and has a greatly varying molecular weight which can range from 1 kDa to 10,000 kDa [197]. Like other GAGs, due to its negative charge, HA is able to bind large amounts of water which allow it to function as a space filler and biological lubricant of joints [198]. HA based applications have been used since the 1980s as a surgical aid for eye-related surgery. Since then it has been used in viscosupplementation treatments, where HA is injected to supplement synovial fluid in (osteo)arthritic joints [199]. Early in the 2000s, HA became a popular ingredient for cosmetic surgical applications like injectable fillers for facial wrinkles. In the last decade, HA has enjoyed gaining popularity as a biomaterial due to its biocompatibility and biodegradability in combination with its gel-forming capabilities. HA can be crosslinked directly using formaldehyde or divinyl sulfone to form a weak but stable hydrogel. However, HA can also be modified by attaching thiols, methacrylates and tyramines to create self-gelling systems [200, 201]. HA is also a component frequently used in combination with fibrous proteins like fibronectin and collagen [202].

3.2. Non-mammalian-based ECM Materials

The biomaterial armamentarium is filled with many non-mammalian raw materials isolated from sources such as insects, (shell)fish, plants and microorganisms. In this section a selection of frequently used biomaterials is given, most of which are based on polysaccharides [203]. Countless mammalian proteins that have been successfully produced in transgenic expression systems, using both mammalian and non-mammalian hosts, are omitted in this review.

3.2.1. Silk Fibroin

Silk is a naturally occurring protein polymer produced by a wide variety of insects and arachnids [204]. The natural function of silk is generally to make webs, cocoons and

draglines. In its natural form, silk is composed of a filament core protein, silk fibroin, and can be coated with glue-like sericin proteins [205]. The composition of different silk types vary depending on the organism and subsequent function [206]. However, all silks have a similar structure composed of hydrophobic and hydrophilic blocks. The hydrophobic blocks are highly conserved and have a repetitive sequence of short-side chain amino acids. The hydrophilic blocks form β-sheet structures and have more complex sequences that consist of larger side-chain and charged amino acids. The combination of the hydrophobic and hydrophilic blocks determines the mechanical properties [207]. It is due to the exceptional mechanical properties, that researchers in the field of biomaterials have gained great interests in harnessing its capabilities [208]. Additionally, depending on the exact species from which it is harvested, silk is thermostable up to 250°C which is extremely high for a protein structure [209]. Isolated silk fibroin is soluble in aqueous conditions which makes it compatible with many processing techniques [210]. The techniques used in combination with silk fibroin, ranges from simple casting and drying methods [211] to hydrogel formation [212] and from electrospinning [213] or fiber deposition [214] to microporous scaffolds made by salt leaching, gas foaming, freeze drying or freeze-thawing [215-217]. For further reading, please see elaborate reviews by Wang *et al*., Altman *et al*., Harkin *et al*. and Vepari *et al*. [208-210, 218].

3.2.2. Alginate

Alginates (also referred to as algin or alginic acid) comprise a broad family of polydisperse linear anionic polysaccharides naturally found in brown seaweeds and consist of varying sequences of β-D-mannuronic acid (M-blocks), α-L-guluronic acid (G-blocks) or mixed sequences (MG-blocks) [219]. Typical alginate size ranges from 500 – 5.000 residues per chain (100 – 1000 kDa) [220]. Alginate can chelate with divalent cations which results in ionic bond driven gelation, also referred to as the “egg-box-model” [221]. The interaction is mainly driven by interaction of the G-blocks that form stronger bonds with the divalent cations than the M-blocks. Depending on the seaweed source and growing conditions, the ratio of mannuronic and guluronic acid can vary, thereby affecting the alginate biomaterial properties [222]. At this moment the exact sequence of alginate, if any, is unknown, and the differences can only be expressed as average values of amounts of the different M/G-block combinations.

Mainly due to the simplicity of the ion driven gel-forming capacity under physiological conditions in combination with its low cytotoxicity, the biomaterial community has

taken great interest in alginate [223]. Alginate is versatile and can be subjected to other processing methods including, but not limited to, freeze-drying [224], electrospinning [225], 3D printing [226], and other chemical modifications or crosslinking methods like, phase transition [227], cell crosslinking [228], click reactions [229], and free radical photo-polymerization [230]. Moreover, due to its gelling properties, alginates are highly useful in the design of microspheres for delivery of cells, growth factors, genes and other drugs [231, 232]. A vast array of alginate-based scaffolds and other devices have thus been made, however most cells require peptide sequences to attach to certain surfaces (see figure 4). Since alginate is a polysaccharide it may require modification for certain cells to be able to adhere [233]. This can be achieved by attaching RGD or other peptides sequences to the alginate chains using carbodiimide crosslinking [234]. This technique couples the terminal free amine group of a protein sequence to the

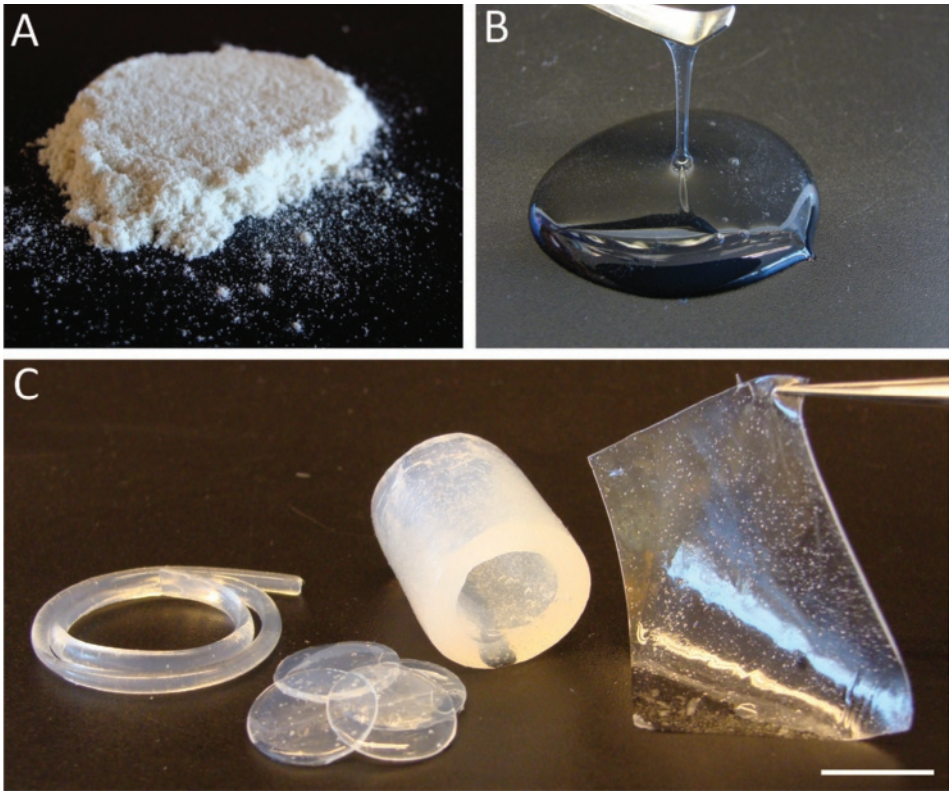


Figure 4: Alginate biomaterials. A) Commercial alginate powder. B) A 5% (w/v) alginate in phosphate buffer results in a viscous gel-like solution. C) Various scaffold shapes can be prepared by exposing the alginate solution to divalent cations, ranging from tubes and gels to films and massive strands, bar is 1 cm.

carboxylic group in alginate [235]. To further enhance the biocompatibility, alginate has been extensively used in combination with other biomaterials like decellularized ECM powder [236], GAGs [237], collagen [238] and other carbohydrates [239]. For further details regarding the extensive subject of alginates the readers are referred to several reviews [240-242].

3.2.3. Chitosan

Chitin can be found in natural sources ranging from invertebrates, fungi, algae and yeasts [243]. Chitosan is a linear polysaccharide derived from the partial deacetylation of chitin and is composed of N-acetyl D-glucosamine and D-glucosamine units [244]. The deacetylation of chitin can vary from 40% to 100% and the molecular weight depends on the source and preparation method (300 to 1000 kDa) [245]. The deacetylation is generally achieved by the use of chemical (alkaline) or enzymatic hydrolysis [246]. The chitosan properties can be affected by the degree of (de)acetylation which decreases the acetyl groups and increases the number of free amine groups on the C2 or C5 position of the D-glucosamine molecules. 40% of the acetyl groups in chitin should be removed before it can be referred to as chitosan [247]. The resulting free amine groups in the chitosan structure can be protonated, making it more soluble than chitin at slightly acidic conditions [248]. The free amine groups makes chitosan a positively charged polysaccharide [249]. Additionally, since amine groups are often involved in ionic bonds and formation of covalent peptide bonds, it makes chitosan suitable for hybrid products with other protein-based and synthetic biomaterials [250].

Numerous studies focusing on chitosan and chitosan hybrid materials have indicated low cytotoxicity, biocompatibility and biodegradability [251]. These properties have attracted attention from the biomaterial community and have led to study of various applications in for example drug delivery [252], gene therapy [253], RM [254] and hemostatic applications [255]. For more detailed overviews please see these reviews [244, 256, 257].

3.2.4. Other polysaccharides

Next to the larger class of biomaterials being used for RM purposes, like GAGs, alginate and chitosan a group of polysaccharides such as dextran and cellulose, remain underutilized. The biological activity and biocompatibility of polysaccharides have been demonstrated in other applications dealing with various cell culture applications. However, polysaccharides are attracting attention due to their capability to form

hydrogels. Depending on the polysaccharide the hydrogel characteristics can vary and have uses in different areas. Glucose-based polysaccharide biomaterials include starch, dextran and cellulose, where the differences are noticed in the way the glucose molecules are bound or if they are linear or branched. Dextran has long been used as an antithrombotic agent by reducing the viscosity of blood. However dextran has also been mixed with other biomaterials to modify the gelation properties for scaffolds used in an array of applications [258-260]. Starch has also found its way as an ingredient of biomaterials that modify the stiffness of the scaffold in question [261, 262]. Cellulose is naturally a very strong and versatile polysaccharide and, due to its crystalline structure, has been mainly used for hard tissue engineering [263]. More complex polysaccharides like agarose and pectin consist of less ubiquitous saccharides like D-galactose and derivatives thereof in the case of agarose. Pectins consist of mainly D-galacturonic acid with appendant residues such as D-xylose, D-adipose and D-arabinose. Both agarose and pectin are slowly starting to find its way into biomaterials for RM [85, 264, 265].

4. Techniques and major tools for scaffolding

Running parallel to the emergence of new biomaterial ingredients is the development of techniques to produce scaffolds or tissue engineered constructs from these very same ingredients. In comparison to decellularized materials, molecularly defined materials can be transformed into a wide array of shapes and sizes, often with a controlled morphology. This is also the main advantage in using molecularly defined ingredients, where the morphology can be adjusted to the application in question. Moreover, in contrast to decellularized materials, the increase in standardization and reproducibility should eventually lower the cost of production and thus the eventual treatment modality. In general, molecularly defined scaffolds possess morphological characteristics having a porous, hydrogel or fabric-like structure [266]. All types of scaffolds have their own advantages and limitations [4]. Ideally, the mechanical properties of the biomaterial in question should match those of the target tissue but should simultaneously passively or actively stimulate cell growth, migration and differentiation. Often an optimum between porosity and the correct mechanical properties is sought after. The porosity is important to the functioning of the scaffold because it impacts the ability of the cells to migrate and nutrients to diffuse throughout the scaffold. In this section different tools are described which can be used to create scaffolds from natural ECM-based sources with control over the morphology.

4.1. Porous Materials

Porous materials represent one of the largest classes of molecularly defined scaffolds currently being researched. Methods and fabrication technologies to produce porous scaffolds are widely available for both natural and synthetic polymers [267, 268]. Next to the obvious requirement that the scaffold should support cell growth, the idea behind the use of porous scaffolds consists of two main reasons where the pores should facilitate cell infiltration and the supply of nutrients. By inducing pore-like structures, the surface area of the scaffold is greatly increased and should subsequently allow for cells to create their own microenvironment. An important requirement inherent to porous scaffold is the interconnectivity of the pores, which absence would hamper cell infiltration and nutrients diffusion [269]. Next to the porosity and interconnectivity, the shape and size of pores are important to accurately mimic the target tissue. In the body, ECM structures often have special arrangements or alignments to cope with a certain function. Depending on the application, porous scaffolds have been used for countless *in vitro* and *in vivo* RM studies. In the case of cartilage tissue, spatial differences in collagen fiber orientations provide the tissue with its properties: in the superficial

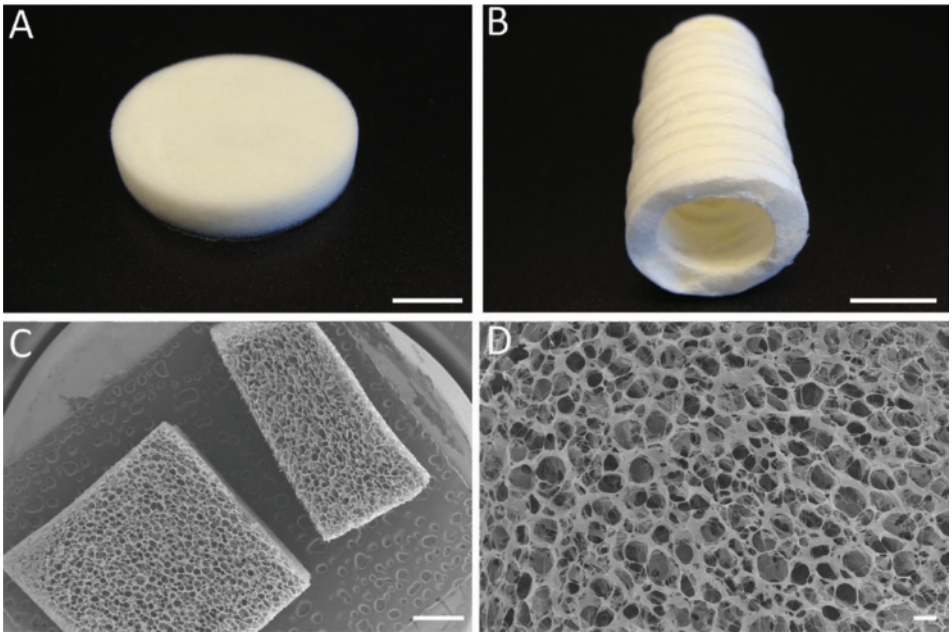


Figure 5: Porous scaffolds. A) Basic collagen scaffold made using a standard 6 well plate, freezing and subsequent freeze-drying, bar is 1 cm. B) Collagen scaffold made in similar fashion but with a different mold consisting of a tube as outer container and a mandrel to create the lumen, bar is 1 cm. (C,D) SEM images of a basic porous collagen scaffold made using freezing and freeze-drying, bars are 1 mm in C and 100 µm in D.

zone collagen fibers are arranged parallel to the articular surface to optimally resist shear stresses during joint loading. In contrast, the orientation of collagen fibers in the deep zone is perpendicular to the articular surface, providing compressive strength to the tissue [270]. To impart porosity on biomaterials, e.g., porogens in combination with solvents [271], and phase separation in combination with solvent evaporation (precipitation [272], gas foaming [273], salt leaching [274], freezing and lyophilization [275]) are used. Novel techniques are emerging to make porous scaffolds, like stereo- and photolithography [276, 277]. An advantage of these processes is that they are promiscuous techniques, compatible with different materials and is easily adapted for straightforward scaling-up and subsequent lowered cost of production.

Using collagen as an example, different porous scaffold types can be made using homogenization and subsequent freezing processes (see figure 5) [278]. The shape into which the collagen gel was cast prior to the freezing process mainly determines the final

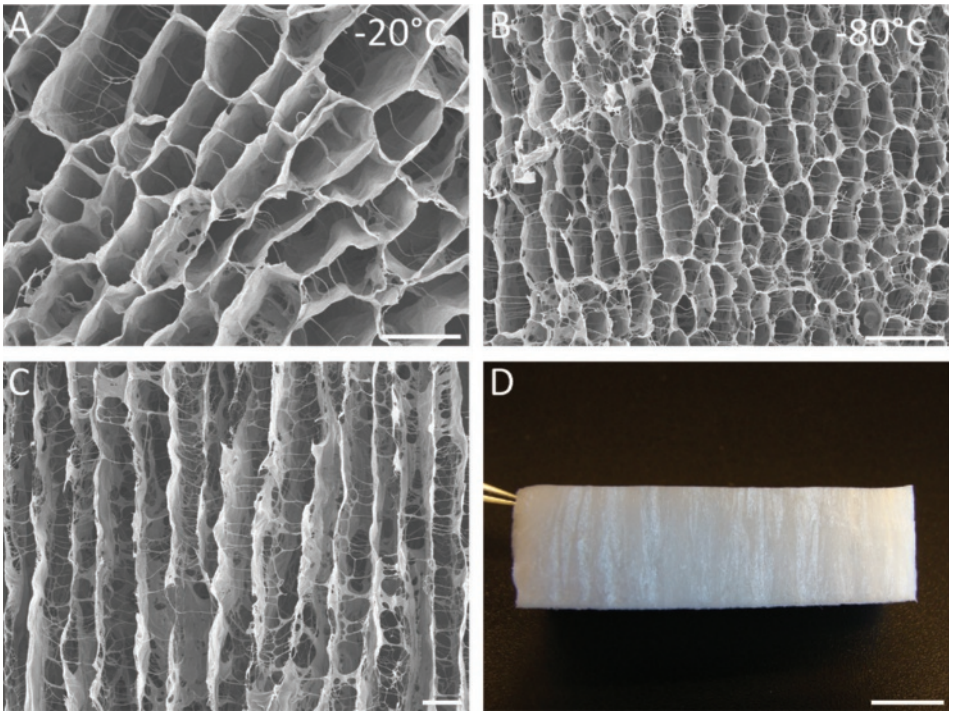


Figure 6: Controlling pore structure. A) Influence of temperature on pore size of a collagen scaffold frozen at -20°C and at B) -80°C, both bars are 100 µm. Microscopic C) and macroscopic D) appearance of a unidirectional collagen scaffold where the direction of the pores was controlled using directional freezing, pores are oriented from bottom to top. Bars are 100 µm and 1 cm, respectively.

macroscopical morphology of a collagen-based porous scaffold. The ultrastructure of a porous scaffold can be created with freezing and freeze-drying using most ECM-based materials, which are either soluble or easily dispersible. Materials less compatible with this method may require other methods to impart porosity as previously mentioned.

The freezing temperature influences the formation of ice crystals (which become pores after lyophilization), including their size and orientation (see figure 6) [279]. In several tissues, the ECM is oriented either unidirectionally (e.g. cartilage) or radially (e.g. muscle fibers in the diaphragm). A scaffold with unidirectional pores can be created by applying a vertical temperature gradient to a collagen suspension (see figure 6), which mimics the ECM of healthy cartilage [278]. A similar principle can be applied to guide the generation of muscle cells in the correct orientation using a scaffold with a radial pore structure. This can be achieved by applying directional freezing from inside-out using a centrally positioned cooled tube [280].

4.2. Hydrogels

Hydrogels are defined as insoluble, three dimensionally (crosslinked) polymer network structures composed of hydrophilic homo- or hetero-co-polymers, which have the ability to absorb significant amounts of water [281, 282]. As in porous biomaterials, hydrogels can be made from different natural and synthetic homo- and copolymers in combination with a variety of preparation methods. A key advantage of hydrogels is the preparation versatility, wherein the suspension can pre-seeded with cells before the hydrogel is formed, which results in homogeneous distribution [283]. The gels can be made using many materials e.g. collagen molecules [284], gelatin [285], elastin [286], alginate [287], silk-fibroin [288], cellulose [289], chitosan [290], hyaluronic acid [291] and others [58, 292]. However, hydrogels are generally soft structures lacking the mechanical properties necessary for many load bearing TE applications [293]. Hydrogels are mainly classified according to their physical structure; amorphous, semi-crystalline or hydrocolloidal [294]. The formation of the hydrogels is based on different techniques; covalent bonds produced by the reaction of one or more co-monomers, freeze-thaw cycles, physical crosslinks due to chain entanglement or enzymatic reaction, association bonds including hydrogen bonds or strong van der Waals interactions between chains, crystallites bringing together two or more macromolecular chains and *de novo* fibril auto assembly [295, 296]. Adjusting parameters like concentration, temperature or the application of shear forces or magnetic fields during the polymerization process (hydrogel formation) can influence

the structure [284, 297]. For further reading the following reviews are recommended [298-300].

4.3. Films and Coatings

Natural ECM components can be processed into thin films or coatings for other (bio) materials. Several applications might require scaffolds, which are non-porous or, even impenetrable for liquids and/or gases. Films are generally regarded as thin, non- or low-porous scaffolds with interesting mechanical properties [301]. Coatings can often have the same composition as films, but are mainly intended to add certain properties, like hydrophilicity and biocompatibility, to other (bio)materials [302]. The film components can be varied and used in different combinations to adjust its properties like transparency, strength, elasticity, nano-porosity, biodegradability and biocompatibility [303, 304]. The science behind design and production of films from natural ECM components is often based on the knowledge gathered from synthetic or natural polymeric films [305, 306]. Synthetic film formation is frequently based on melting/dissolving and subsequent solidification of the polymer. However, the use of polysaccharides and relatively fragile proteins call for different approaches that avoid high temperatures and corrosive conditions. Soluble proteins and polysaccharides are often compatible with an array of techniques such as dip coating, spray coating, spin coating, or solvent casting and subsequent drying to produce films or to coat existing

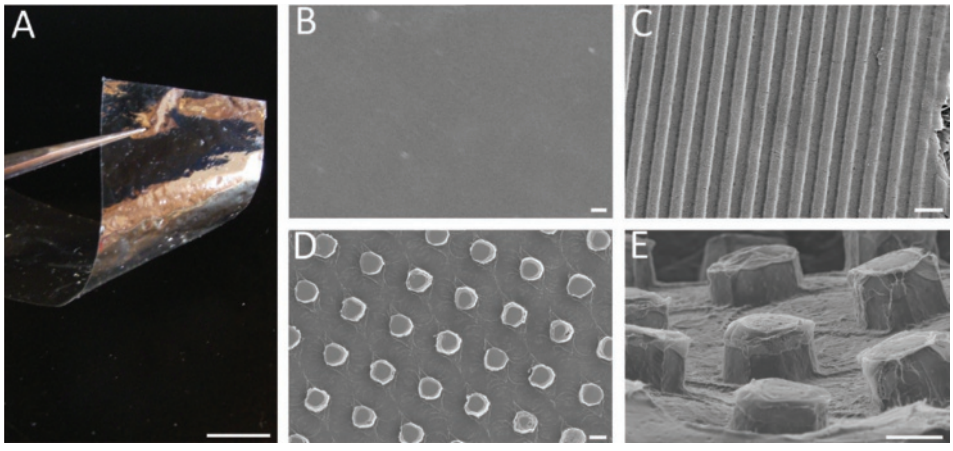


Figure 7: Films and topography. A) Macroscopical image of a collagen film made by air-drying, bar is 1 cm. B) SEM image of a collagen film, bar is 1 µm. C) Surface topography induced by varying casting shape, bar is 1 µm. (D,E) Another possible variation of surface topography, made to mimic the rete-ridge structure between the dermis and the epidermis, bars in D,E are 100 µm [314].

(bio)materials. Films have been made from many different raw materials including, but not limited to collagen [307], gelatin [308], keratin [144], silk [209], alginate [309] and chitosan [301]. Due to their high transparency, films have frequently been used in TE of the cornea [310], but also for nerve regeneration [311], engineering of skin and adipose tissue [312, 313]. In some applications the films require certain structure or topography to stimulate directional growth and differentiation of the cells (see figure 7). Techniques have been developed which can induce structure using micro-molding [306], casting [314], and laser patterning [315]. For further details the readers are referred to reviews by Chen *et al.* and Wibowo *et al*. [316, 317].

4.4. Meshes: Spinnings, knittings, and windings

Synthetic polymer-based biomaterials are highly compatible with techniques that create meshes using for example electrospinning or fabric-based techniques like weaving, knitting or winding (see figure 8) [318]. Using biomaterial meshes in construct design has several advantages with respect to defined fiber alignment, adjustability, reproducibility and versatility. Synthetic polymer meshes have been readily used in the surgical arena for decades. Surgical meshes or knittings represent a group of implants mainly used for heavy-duty tissue repair, like herniations in the abdominal wall. Two major mesh concepts are distinguished, the classical concept including so-called heavy weight meshes with small pores and the newer concept including light weight meshes with large pores [319]. Techniques used to make these meshes often require corrosive chemicals and high temperatures, which can render them unsuitable for use with natural ECM components [320]. However, in the recent years, developments in this area have resulted in several options to make meshes from more natural ECM components. Electrospinning has received bulk of the attention. It is based on the difference in (electrical) potential applied to a negative or positively charged solvent containing the polymer and a oppositely charged collector surface during extrusion [321]. During this process different fiber dimensions can be formed and deposited on wide array of surfaces to create an interconnected network or a mesh [322]. Electrospinning has been used with natural ECM components like soluble collagen, gelatin, chitosan, chitin, cellulose and starch [323]. Due to the interchangeability of the collector surface and the possibility to rotate the surface during the electrospinning process allows for control over the fiber direction in 2D meshes and 3D tubular constructs [322, 324]. Other methods to create meshes from natural ECM components are often two-staged where first a fiber or strand should be extruded which can subsequently be processed into fabric-like meshes [325].

Not all biomaterials can be extruded into a strand that is mechanically stable enough to undergo further processing [112, 326]. However, if a stable strand is formed, it can be turned into a fabric using knitting, weaving or winding. Depending on the target tissue, one can opt for making a flat mesh using knitting or weaving [327] and tubular meshes can be made using adapted knitting techniques or winding methods [328]. Different mechanical properties can be induced using different knitting, weaving and winding patterns. For example using the stockinette knitting technique, which induces anisotropic rigidity, tissues requiring particular mechanical properties in certain directions can be partially mimicked [329].

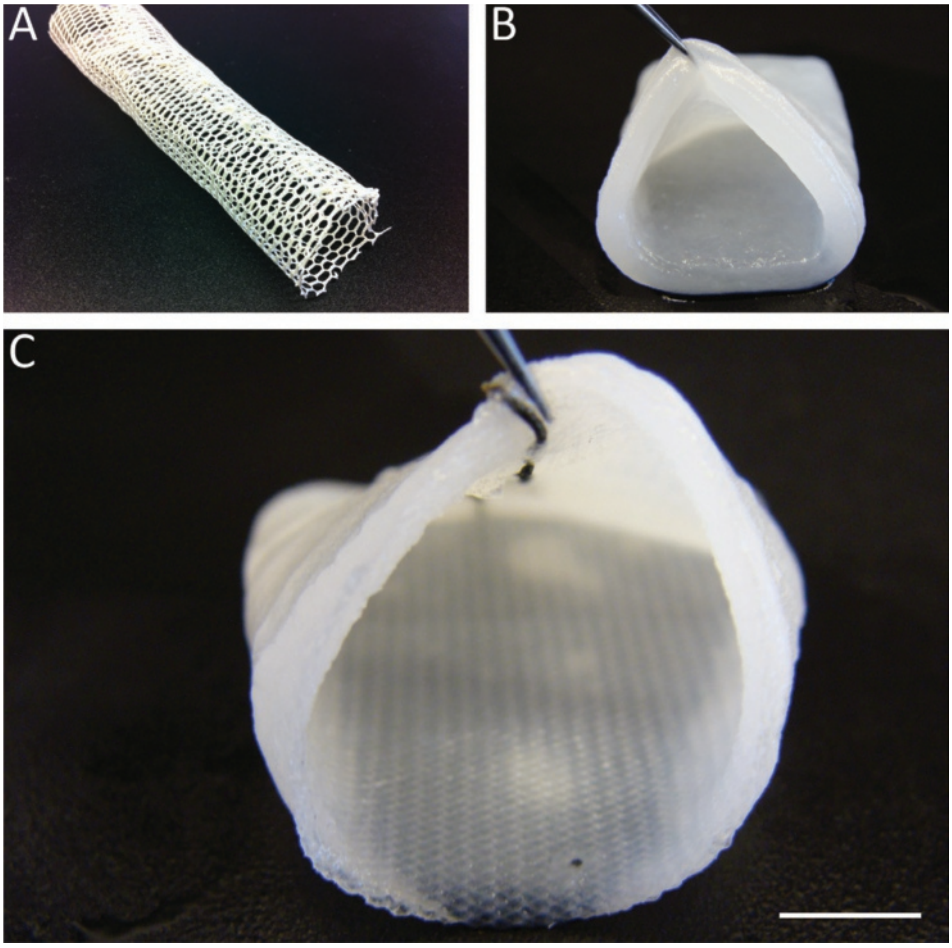


Figure 8: Applications of knitted polymers in collagen scaffolds. A) A tubular knitting can be prepared from any biomaterial (in this case a synthetic polymer, polycaprolactone) that can be processed into a flexible strand. B) A collagen tubular collagen scaffold without reinforcement. C) A collagen scaffold where a biodegradable knitting was incorporated, bar is 0.5 mm.

4.5. Computer controlled fabrication

Parallel to the rapid advancements in the field of medical imaging, where high-resolution 3D images of a defect can be easily acquired, runs the development of rapid prototyping techniques [330]. Rapid prototyping allows for the medical imaging data to be processed with computer-aided design and subsequently automatic manufacturing of three-dimensional objects layer-by-layer according to the virtual design. The utilization of rapid prototyping in RM enables the production of 3D scaffolds with intricate geometries and very detailed structures [331]. The resulting scaffolds can be customized and made to match each patient's individual need [332]. Major rapid prototyping methodologies include 3D printing [333], multi-jet modeling [334], stereo-lithography [335], selective laser sintering [336] and fused deposition modeling [337]. These techniques are very promising for hard tissues since they are generally compatible with synthetic polymers that often require high temperatures and corrosive solvents [338]. Basically any form of lithography requires liquid-based materials which are sensitive to photo-polymerization [339]. Laser sintering and fused deposition techniques are generally based on temperature driven fusion (melting), which excludes the use of some natural ECM components. Up until now, printing techniques seem to be compatible with natural ECM components where researchers have managed to print for example collagen [340], gelatin [341], silk fibroin [342] and alginate [343]. Moreover, printing technology is also capable of producing patterns using bioactive ingredients such as growth factors [344]. In general, printing of the aforementioned biomaterials is less developed compared to the printing of synthetic polymers. Currently, problems like low resolution and low mechanical strength are being addressed. However along with the emergence of printing natural ECM components came the realization that the whole process could be conducted under aseptic/sterile conditions allowing for the addition of live cells.

Many variations have been developed but the technique is often referred to as 'bioprinting' or 'cell printing' [345]. The printing of single cells using common inkjet printer technology was useful for printing monolayers. Consequently, early efforts have focused on tissues like the skin where flat constructs were printed layer-by-layer using subsequent monolayer of cells and a hydrogel of choice [346]. A logical step forward encompassed the printing of cell aggregates or spheres. In this technique cells, in magnitude of thousands, are encapsulated in a hydrogel sphere and is subsequently printed into another hydrogel which keeps the spheres in place [347]. During culture the spheres fuse and the cells subsequently arrange in tissue like formations, subsequently

circumventing the need for high-resolution printing [348]. More advanced bioprinting techniques have been developed by switching from the inkjet principle to the laser printer generation technology, known as laser-assisted bioprinting [349]. More variations on computer aided scaffold production are emerging with each method baring its limitations. For more information regarding biomaterial printing the reader is referred to several reviews [350, 351].

4.6. Decellularization

Biomaterials derived from natural tissues can be referred to as decellularized matrices or extracellular matrix scaffolds. This scaffolding strategy is based on the removal of cells and cellular antigens from the allogeneic or xenogeneic tissues (see figure 9). However, the ECM components, which should be well tolerated by the immune system such as collagen, elastin, glycosaminoglycans and are preserved [352]. Over the past decades many decellularization methods have been developed [353]. These cell extraction methods can impart chemical, physical and enzymatic treatments or a combination thereof. Chemical treatments are aimed at disrupting the cell membranes and intra- and extracellular structural proteins. Frequently used chemicals include detergents [354] e.g., Triton x-100 [355], sodium deoxycholate [356] and sodium dodecyl sulfate [357]. Also acids [358] and bases [359], hypo- and hypertonic solutions [360], organic solvents [361] and chelating agents [362] can be applied in decellularization procedures. Enzymatic treatments are mainly based on enzymes possessing protease

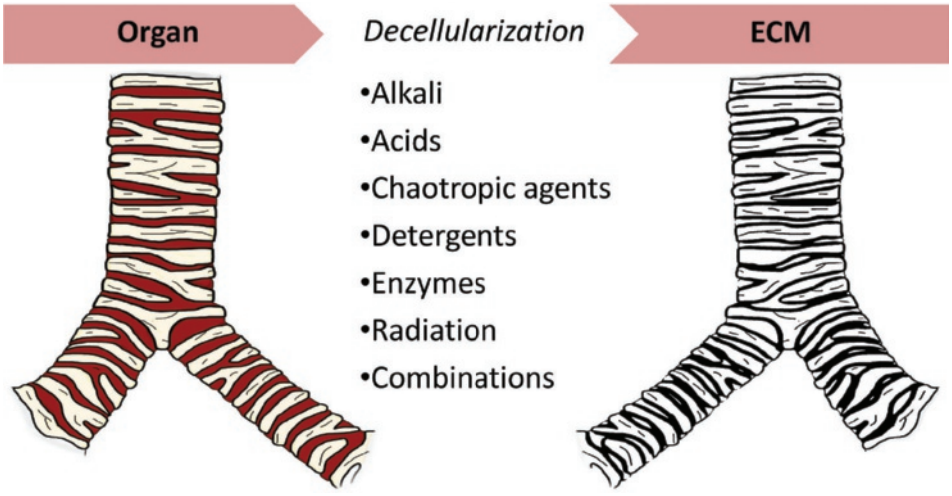


Figure 9: Methods to obtain decellularized scaffolds: Cadaver organs, in this illustration a trachea, are exposed to a wide-array of chemical, biochemical and physical treatments aimed at removing all cellular components and unwanted proteins, effectively rendering native ECM.

and nuclease activity like for example ones falling within the family of trypsins [363], DNAses [364] and RNAses [365]. Physical methods to remove the cellular components can include sonication [366], high pressure [367], super-critical CO_2 [368], freeze-thawing [369], agitation [370], and mechanical abrasion [371].Tissues currently under investigation include but are not limited to pericardial membrane [372], trachea [373], blood vessel [374], kidney [375], tendon and ligament [376], heart [377] and heart valve [378], cornea [379], liver [380], small intestinal submucosa [381], lung [382], nerve [383], esophagus [384], bladder [385], adipose tissue [386] and skin [387].

The advantage inherent to decellularized materials is that they can be used to generate a largely identical structure and composition of extremely complex target tissues. Depending on the method of isolation, certain components may or may not be targeted for removal. For example the use of bases like sodium hydroxide are frequently used as a virus inactivation step, however also tend to inactivate growth factors and remove GAGs. Detergents like Triton X-100 and sodium dodecyl sulfate (SDS) effectively remove nuclear remnants and cytoplasmic proteins. Decellularization methods have thus to be carefully selected to balance the scaffold requirements with safety and efficacy. Affecting the collagen structure and GAG content may compromise mechanical integrity of scaffolds [388]. Decellularization agent remnants may have a toxicological effect on the cells, affecting the treatment efficacy [389]. Incomplete decellularization of the tissues may lead to the increased immunogenicity, *ergo*, increasing the possibility of eliciting an immune response [390-392]. Moreover, decellularized tissues are by nature highly variable as is the production process and it is difficult to show complete decellularization without compromising the structural integrity of the construct.

Nonetheless, decellularized tissues make up a large part of the commercially available products. Several examples of FDA approved commercially available decellularized ECM scaffolds include; AlloDerm, AlloPatch, NeoForm and Graftjacket (all human dermis), Permacol, Strattice and Zimmer Collagen Repair Patch (all porcine dermis), TissueMend (fetal bovine dermis), MatriStem (porcine bladder), CuffPatch, OaSIS, Surgisis, Restore, FortaFlex and CorMatrix ECM (all porcine small intestinal submucosa), IOPatch (human pericardium), OrthAdapt and Unite (equine pericardium), CopiOs, Lyoplant and Perimount (all bovine pericardium) and, Hancock II, Mosaic, Freestyle, Prima Plus, Epic and SJM Biocor (porcine heart valve) [353]. The majority of the commercialized constructs are utilized in heart valves [393], dentistry [394], chronic wounds [395] and soft tissue reconstruction in general (hernias, burn wounds, organ

slings, etc.) [396, 397]. For further reading in commercially available biomaterials used in RM please see the excellent review by Keane *et al*. [183].

4.7. Crosslinking

Many of the described natural ECM-derived biomaterials can be subjected to a wide array of processing methods to create a 3D construct or environment. However, depending on the biomaterial type, the construct can be mechanically unstable and subject to rapid degradation. Using crosslinking techniques to enhance the mechanical and enzymatic resistance, properties of a biomaterial can be stabilized for use at physiological conditions and subsequent implantation purposes. In its essence, crosslinking is defined as the formation of bonds within or between the biomaterial subunits in question [398]. In general, the bonds formed are mostly covalent, however in the case of alginate or chitosan, inducing ionic bonds using polycationic molecules can also be regarded as crosslinking [221].

Since the majority of the natural ECM-derived biomaterials are protein or carbohydrate based, many of the developed crosslinking techniques are based on creating bonds between reactive groups like carboxylic groups and amines. Other reactive moieties include but are not limited to sulfhydryl, hydroxyl and carbonyl groups. In general there are three types of crosslinking processes: physical, chemical and enzyme-based crosslinking. Physical crosslinking methods rely on either irradiation or the use of high temperatures. Irradiation can induce free radicals that in turn react with other chemical groups near by [399]. Irradiative sources mainly refer to ultra-violet wavelengths (UV) but also to gamma and beta irradiation [399, 400]. Thermal sources in combination with high vacuum can be used to induce the dehydrothermal crosslinking which allows for formation of covalent bonds [401]. Enzymatic crosslinkers like transglutaminase can be used to enhance tensile strength and enzymatic resistance of collagen-based biomaterials [402]. One should take into account that the enzyme should also be inactivated and subsequently removed from the biomaterial in most cases. The use of enzymatic crosslinkers can eliminate the risk of inducing cytotoxic effects [403]. The largest and most diverse group of crosslinkers are the chemical crosslinkers. Glutaraldehyde along with other aldehyde-based chemicals are the most applied chemicals to crosslink protein-based biomaterials [404]. The carbodiimide family is another class of chemicals used to induce crosslinking [405]. A key feature of this method is that some carbodiimides are categorized as zero-length crosslinkers. For example EDC (1-ethyl-3-(-3-dimethylaminopropyl carbodiimide hydrochloride) directly

couples primary amine groups to carboxylic groups without introducing a linker that may elicit an immune response (see figure 10). A less known member of the chemical crosslinkers is the isocyanate chemical family [406]. Recently, genipin a chemical crosslinker derived from fruit extracts, has shown potential because of its low toxicity [407]. In contrast to enzymatic crosslinkers, certain chemical crosslinking techniques can potentially form toxic residues or create crosslinks and subsequent metabolic products non-native to the human body [408, 409].

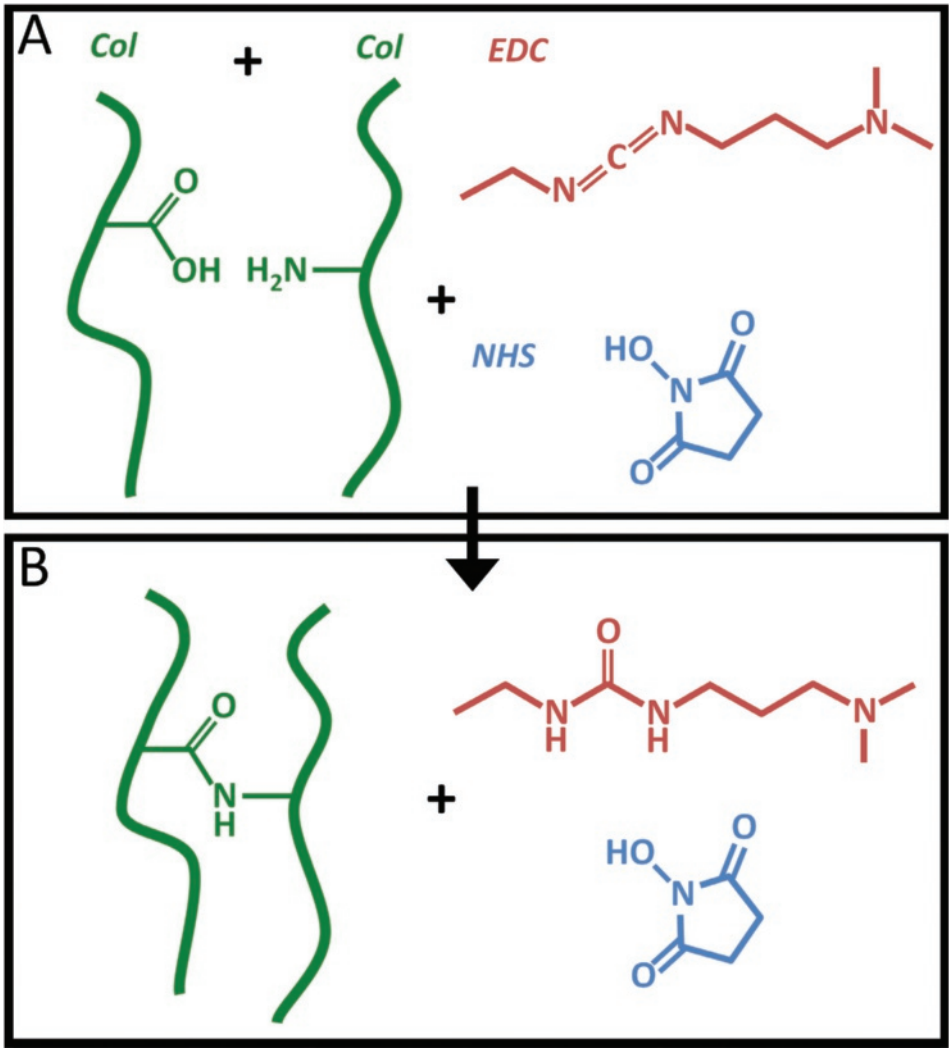


Figure 10: Crosslinking collagen using EDC and NHS: (A,B) EDC and NHS catalyze covalent bindings between carboxylic acid and amine groups. Col: collagen. EDC: 1-ethyl-3-(-3-dimethylaminopropyl) carbodiimide hydrochloride (EDC). NHS: N-hydroxysuccinimide.

4.8. Sterilization of biomaterials

Medical devices, such as ECM-based scaffolds, are made to be implanted in the patient. To avoid the risk of introducing microbes, viruses or other pathogens, sterilization is necessary. The sterilization process aims to reduce the amount of viable pathogens to an acceptable standard, since 100% sterility can never be guaranteed. The worldwide accepted standard for sterility of implantable medical devices is defined as the chance of finding a viable micro-organism in or on a medical device to be at most 10^{-6} [410]. Other pathogens like viruses, endotoxins or prions are not considered as micro-organisms, and are difficult to remove. However, biomaterials treated with sodium hydroxide can reduce the viral load and decrease the chance of prions infection [411]. In this respect, it is important to reduce the risk of introducing these pathogens during the production processes of the medical device. This can be achieved by using animal sources that originate from strictly controlled environments, specifically aimed at preventing contact with specific pathogens. For instance, for bovine-derived materials, the EDQM (European Directorate for the Quality of Medicine & HealthCare) and FDA (Federal Drug Administration, USA) recommend the use of closed-herd cattle from countries that have a low-risk for bovine spongiform encephalopathy (BSE) such as Australia or New Zealand [412, 413]. Next to that, the production process of the ECM-based scaffold should be carried out in cleanroom facilities and under good manufacturing practice (GMP) guidelines. Next to these precautions, sterilization remains required to ensure acceptable sterility assurance level (SAL) values. Preferably, sterilization takes place after packaging to reduce the risk of contamination after sterilization.

Sterilization methods are based on the application of an external stimulus, such as heat, ionizing radiation or reactive chemicals, that kills or inactivates the micro-organisms, leaving the medical device functional. For medical instruments, heat sterilization (autoclaving) is often applied. However, biomaterials are not always resistant to heat, especially protein-containing materials are sensitive to high temperatures and therefore not autoclavable. Alternative sterilization methods based on chemicals or radiation are required for biomaterials. In this section the strengths and limitations of both approaches will be discussed in addition to highlighting some recent developments.

4.8.1. Chemical-based sterilization

One of most frequently applied forms of chemical sterilization is the use of ethylene oxide gas. Microbiological inactivation by ethylene oxide is based on the alkylation of hydroxylic, amine and carboxylic groups of cellular components such as DNA or proteins [414]. The ECM-based scaffolds themselves can also be affected by this powerful sterilizing agent. A recent study of Matuska and McFetridge showed that ethylene oxide caused unfavorable structural damage to decellularized collagen scaffolds [415]. In addition, ethylene oxide can change the enzymatic degradation rate of collagen, although variable results have been found, e.g. decreased degradation of dermal sheep collagen [416] versus no effect on bovine type I collagen scaffolds [417]. Oxidizing agents such as peracetic acid or hydrogen peroxide are known for their efficiency to kill microbes by denaturing proteins and disrupting cell membranes [418]. An advantage of these sterilizers, frequently used together, is that they require a much shorter cycle time compared to ethylene oxide [419]. In addition, both have a low residual toxicity and degrade into non-toxic products, namely water, oxygen and carbon dioxide [420].

The removal of remnants of chemical sterilization from the scaffold is an important issue that should be taken seriously when applying chemical sterilization. As shown by Markowicz *et al*., hydrogen peroxide remnants in porous collagen scaffolds could still be detected after gas plasma sterilization [421]. For ethylene oxide, it is also known that remnants can be detected in medical devices after sterilization [422]. For each type of biomaterial, the bioavailability of the hazardous remnants must be evaluated.

Recent developments in the field of sterilization focus on the improvement of the delivery and removal of chemical agents in and out of the medical device. Supercritical carbon dioxide may provide an alternative means to deliver chemical sterilizers inside scaffolds. This supercritical fluid possesses physicochemical characteristics in between a gas and a liquid and therefore exhibits excellent diffusion properties [423]. It has been shown that supercritical carbon dioxide in combination with hydrogen peroxide could completely sterilize highly porous collagen sponges [424]. Another interesting physical-chemical technique is gas plasma sterilization. With this technique, a substance is brought into the gas phase under vacuum. Next, an electric field is applied on the gas resulting in the formation of reactive species, which cause microbiological inactivation. In the pure form of this technique, the gas itself has no biocidal effect unless it is activated by the electric field [425]. Currently, gas plasma methods are mainly used

in combination with a reactive compound such as hydrogen peroxide or peracetic acid [426]. Gas plasma techniques can be used at low temperatures, do not generate toxic residues and are relatively rapid [427]. In addition, penetration properties of the chemicals are superior compared to their liquid counterpart.

4.8.2. Radiation-based sterilization

Another conventional sterilization technique is the usage of ionizing radiation such as gamma (γ) radiation and e-beam (beta, β) radiation. γ-Radiation is generally known for its efficiency to inactivate microorganisms, however, also for its damaging effect on proteins [417, 428, 429]. Therefore, the scaffold and added bioactive molecules such as growth factors will always be affected. The addition of preservatives such as glucose during irradiation may reduce the side effects of γ-radiation by stabilizing the collagen molecules [430]. Next to γ-radiation, β-radiation (electron-beam) can also be used. An advantage of this technique compared to γ-radiation is that dangerous radioactive sources such as cobalt-60 can be avoided. However, the limited penetration capacity of the β-particles, make this technique less suitable for thick medical devices [431]. X-ray radiation, produced from an electron beam directed on an X-ray converter, could potentially be used to irradiate large packages. The high-energy photons damage living organisms by affecting DNA and other cellular structures. However, this technique is limited by the inefficiency of the conversion of electrons to high-energy photons, which makes the process very costly [411]. Recent developments in high-power and high-energy electron accelerators may make this sterilization approach more attractive [432]. Next to ionizing radiation, microwaves may become applicable in the future. Shamis *et al.* reported the use of non-thermal microwaves for microbial inactivation [433]. Unfortunately, current results with doses that do not affect the biomaterial, are not in compliance with a SAL value of 10^{-6}. Future research may clarify if this method, which avoids hazardous chemicals and radioactive sources, has potential.

4.8.3. Biomaterial-specific sterilization

Despite the efficiency of reactive chemicals and radiation to inactivate microbes, damage to proteins-based scaffolds is inevitable. Moreover, current research in the field of RM is focused on creating bioactive scaffolds that contain fragile molecules such as growth factors. Therefore, recent efforts focus on developing new technologies that are as effective as previously mentioned sterilization methods while avoiding the damaging effect on the biomaterial and proteins. In addition, more environmental-friendly methods, which are easier to use without the need for severe safety precautions, would

be beneficial. Sterilization will always affect the biomaterial and alter the physico-chemical and mechanical properties of the scaffold itself. In some cases, the alterations of the biomaterial can be unfavorable or non-relevant with respect to clinical use, while in other cases it can even be used to modify the scaffold to its specific needs. Andrews *et al*. showed that ethylene oxide sterilization could roughen the surface of an electrospun scaffold and thereby manipulate cell contact, phenotype or function [434]. Also for a collagen-based scaffold, it was reported that sterilization could be used to modulate characteristics to favor cellular adhesion [415]. Furthermore, sterilization can serve as a tool to modify the biodegradability of the ECM scaffold. Kawasaki *et al*. showed that gamma sterilization influenced the bioabsorption time *in vivo* for a porous hydroxyapatite/collagen scaffold [435]. For clinical applications such as biodegradable sutures, gamma sterilization is already used to adjust the bio-absorbability [436]. The sterilization method of choice is highly dependent on the type of biomaterial and the intended application of the scaffold. Consequently, no perfect method exists and researchers have to search for the best alternative for their specific scaffold and application. Furthermore, issues surrounding sterilization should be taken into account early in the developmental process to make sure that the end-product can be sterilized safely and effectively.

4.9. Regulatory Affairs

An important issue concerning biomaterials is a frequent lack of adequate attention early in the R&D process pertaining the regulatory affairs. This complicated pathway can be a huge obstacle in the translation of ECM-based scaffolds to the clinic. Although the fundamental legislation is rather similar, most countries/continents have their own specific legislation, each with their own nuances. In a world of internationally conducted research the differences in legislation make the translational process even more difficult. Below a short overview is given on the regulatory affairs in the European Union and the United States. Acellular scaffolds generally fall within the category of medical devices according to different regulatory agencies. In Europe, medical devices are subdivided into four different classes, respectively I, IIa, IIb and III. Class III has the highest risk regarding safety issues and encompasses all implantable medical devices including scaffolds used in RM [437]. The legislation for medical devices in Europe is established by the European Commission and approved by the European Parliament (Directive 2007/47/EC). The daily execution of the regulation is controlled by so-called “Notified Bodies”, which can be private companies or foundations appointed by the European Commission [438].

A difficulty regarding regulation issues of ECM-based scaffolds is that they are often composed of components derived from animal sources. Derivatives of animal tissues may contain transmissible pathogens such as viruses, bacteria, endotoxins, and prions. Therefore, the European Union (EU) has developed extensive guidelines in order to minimize the risk of infectious disease transmission by medical implants / devices. In this respect, all relevant information about the animal source should be reported, such as -but not limited to- animal species, age, specific tissue used, country of origin, methods for health monitoring of the herd, and transportation condition. The F2027-08 document of the American Society for Testing and Materials (ASTM) provides an excellent guidance for the use of biomaterials for the purpose of RM [439].

Next to detailed information about starting material, all details on the manufacturing process of the scaffold itself, the sterilization procedure, all test methods, test results, and acceptance criteria should be described and provided to the regulatory bodies. International Organization for Standardization (ISO) guidelines can be of great assistance for designing the manufacturing process and aim to ensure that medical devices are safe, reliable and of good quality. The regulation pathway for ECM-based scaffold is rather complex, however if (stem) cells are introduced in the scaffold, this will greatly complicate the regulation progress, since a complex viable entity is added instead of a single or a combination of compounds. For a single compound such as a growth factor, it can already be hard to predict the *in vivo* effects. For a complex cell, it will even be more complicated. In the EU, cellular scaffolds are classified under the advanced therapy medicinal products (ATMPs) and have a different and more complex legislation compared to acellular scaffolds, which are classified as medical devices.

In the US the regulatory system is rather similar to the EU. The regulation for medical devices is controlled by the Food and Drugs Administration, specifically the Center for Biologics Evaluation and Research, a center within the FDA [440]. For further information about the differences in the regulation process between the US and European union, readers are directed to a review by Vinck *et al.* [441]. As the regulation and guideline issues surrounding ECM-based scaffold are very complex, most researchers do not have the proper knowledge to deal with it. To avoid foreseeable regulatory surprises, it is recommended to consult specialists in the field of regulatory issues early in the developmental process. These specialists can help researchers to avoid pitfalls and redundancy, subsequently accelerating the bench-to-bedside application of ECM-based scaffolds.

5. Summary and future perspectives

The ECM of the target tissues/organs not only serves as a structural blueprint but also as a toolkit for tissue engineers. The natural composition of the ECM provides valuable information regarding the properties a final construct or treatment modality should mimic. By examining the role of each ECM component in the natural tissue, engineers can set priorities as pertaining to which components are most important. Major ECM components like collagen, elastin and proteoglycans play important roles in the mechanical and biological properties of the natural tissue. Due to their fibrous nature, collagen and elastin have been used for providing mechanical strength to a construct, in addition to their effects on cell behavior. Depending on the application, the state of the collagen (gelatinous, monomeric, fibrillar or fibrous) and the spatial arrangement greatly affects the mechanical properties. For molecularly defined ECM-based constructs to succeed, control over the properties of the mechanical properties (mainly the collagenous fraction) should be increased. In chapters 3 and 6 of this thesis, new techniques are described to adjust the biomechanical properties that mimic the ECM of the target tissue more closely. In most soft-tissue applications, other components like elastin and GAGs can be added to fine-tune the mechanical properties but will probably not be used in such quantity that it will take over the role of collagen as the main constituent. However, in load-bearing tissues such as cartilage, the water-sequestering GAGs will greatly affect the success rate of collagenous constructs by providing lubrication and shock absorbing capacity, which collagen naturally lacks. Even when the ideal mechanical properties have been attained, it will be important to ensure optimal biocompatibility, where the correct/desired cells are attracted and can subsequently adhere to the construct. Laminin, fibrinogen, fibronectin and type IV collagen are main components of the basal membrane that regulate cell attachment and related processes. Although not covered in this thesis, addition of other components besides collagen may be advantageous for e.g. proper cell attachment.

The true challenge for the tissue engineering society will be to cost-effectively create constructs with the required mechanical properties and bioactive components. Since sterilization of a construct is unavoidable, the mechanical properties should not be affected by this and ingredients should be able to fulfill their respective roles after sterilization. Difficulties caused by sterilization will greatly limit the speed and overall progress of ECM-based constructs in the near future. To make future treatment

modalities possible, knowledge regarding the techniques to produce different constructs is needed. The work described In this thesis focuses on the development of new technologies to prepare novel, collagen-based constructs.

6. Acknowledgements

The authors would like to acknowledge the Microscopic Imaging Center for facilitating electron microscopy equipment (Radboud University Medical Center, Nijmegen, Netherlands). Michiel Pot, Alexey Klymov, Corien Oostendorp, Elly Versteeg, Gerwen Lammers, Katrien Brouwer, Xander van Wijk, Kaeuis Faraj, and Martin Koens are thanked for their research leading up to various images used in this chapter. The research leading to these results has received funding from the European Community's Sixth (EuroSTEC, LSHB-CT-2006-037409), Seventh (Framework programs MultiTERM, 238551 and EuroSkinGraft, 279024), Netherlands Institute for Regenerative Medicine (NIRM, FES0908), and the Dutch Ministry of Economic affairs and Province of Gelderland & Overijssel (Pieken in de Delta program, number PID101020).

8. References

[1] P.A. Corris, J.A. Kirby, A role for cytokine measurement in therapeutic monitoring of immunosuppressive drugs following lung transplantation, Clinical and Experimental Immunology 139(2) (2005) 176-178.

[2] R. Langer, J.P. Vacanti, Tissue engineering, Science 260(5110) (1993) 920-6.

[3] W. Daamen, Preparation and evaluation of molecularly-defined collagen–elastin–glycosaminoglycan scaffolds for tissue engineering, Biomaterials 24(22) (2003) 4001-4009.

[4] B.P. Chan, K.W. Leong, Scaffolding in tissue engineering: general approaches and tissue-specific considerations, European Spine Journal 17(S4) (2008) 467-479.

[5] S.F. Badylak, D. Taylor, K. Uygun, Whole-Organ Tissue Engineering: Decellularization and Recellularization of Three-Dimensional Matrix Scaffolds, Annu Rev Biomed Eng (2010).

[6] L. Cen, W. Liu, L. Cui, W. Zhang, Y. Cao, Collagen Tissue Engineering; Development of Novel Biomaterials, Pediatric Research 63(5) (2008) 492-496.

[7] S. Ozbek, P.G. Balasubramanian, R. Chiquet-Ehrismann, R.P. Tucker, J.C. Adams, The evolution of extracellular matrix, Mol Biol Cell 21(24) (2010) 4300-5.

[8] F.G. Giancotti, E. Ruoslahti, Integrin signaling, Science 285(5430) (1999) 1028-32.

[9] H.L. Birch, C.T. Thorpe, A.P. Rumian, Specialisation of extracellular matrix for function in tendons and ligaments, Muscles Ligaments Tendons J 3(1) (2013) 12-22.

[10] A.J. Engler, H.L. Sweeney, D.E. Discher, J.E. Schwarzbauer, Extracellular matrix elasticity directs stem cell differentiation, J Musculoskelet Neuronal Interact 7(4) (2007) 335.

[11] M. Roth-Kleiner, M. Post, Similarities and dissimilarities of branching and septation during lung development, Pediatr Pulmonol 40(2) (2005) 113-34.

[12] P. Bedossa, V. Paradis, Liver extracellular matrix in health and disease, J Pathol 200(4) (2003) 504-15.

[13] P. Fratzl, Collagen: Structure and Mechanics, 1st ed., Springer, New York, 2008.

[14] V. Ottani, D. Martini, M. Franchi, A. Ruggeri, M. Raspanti, Hierarchical structures in fibrillar collagens, Micron 33(7-8) (2002) 587-96.

[15] L.D. Muiznieks, F.W. Keeley, Molecular assembly and mechanical properties of the extracellular matrix: A fibrous protein perspective, Biochim Biophys Acta 1832(7) (2013) 866-75.

[16] S.T. Nillesen, G. Lammers, R.G. Wismans, M.M. Ulrich, E. Middelkoop, P.H. Spauwen, K.A. Faraj, J. Schalkwijk, W.F. Daamen, T.H. van Kuppevelt, Design and in vivo evaluation of a molecularly defined acellular skin construct: reduction of early contraction and increase in early blood vessel formation, Acta Biomaterialia 7(3) (2011) 1063-71.

[17] J.E. Nuininga, M.J. Koens, D.M. Tiemessen, E. Oosterwijk, W.F. Daamen, P.J. Geutjes, T.H. van Kuppevelt, W.F. Feitz, Urethral reconstruction of critical defects in rabbits using molecularly defined tubular type I collagen biomatrices: key issues in growth factor addition, Tissue Eng Part A 16(11) (2010) 3319-28.

[18] M.J. Koens, K.A. Faraj, R.G. Wismans, J.A. van der Vliet, A.G. Krasznai, V.M. Cuijpers, J.A. Jansen, W.F. Daamen, T.H. van Kuppevelt, Controlled fabrication of triple layered and molecularly defined collagen/elastin vascular grafts resembling the native blood vessel, Acta Biomaterialia 6(12) (2010) 4666-74.

[19] P.J. Geutjes, W.F. Daamen, P. Buma, W.F. Feitz, K.A. Faraj, T.H. van Kuppevelt, From Molecules to Matrix: Construction and Evaluation of Molecularly Defined Bioscaffolds, Adv Exp Med Biol 585 (2006) 279-295.

[20] M.J. Koens, P.J. Geutjes, K.A. Faraj, J. Hilborn, W.F. Daamen, T.H. van Kuppevelt, Organ-specific tubular and collagen-based composite scaffolds, Tissue Eng Part C Methods 17(3) (2011) 327-35.

[21] G. Khang, Biomaterials and manufacturing methods for scaffolds in regenerative medicine, in: G. Khang (Ed.), Handbook of intelligent scaffolds fo tissue engineering and regenerative medicine, Pan Stanford Publishing Pte. Ltd, Singapore, 2012.

[22] C.M. Kielty, M.E. Grant, The Collagen Family: Structure, Assembly and Organization in the Extracellular Matrix,, in: P.M. Royce, B. Steinmann (Eds.), Connective Tissue and Its Heritable Disorders: Molecular, Genetic, and Medical Aspects, John Wiley & Sons, Inc., Hoboken, NJ, USA, 2003.

[23] K. Sato, K. Yomogida, T. Wada, T. Yorihuzi, Y. Nishimune, N. Hosokawa, K. Nagata, Type XXVI collagen, a new member of the collagen family, is specifically expressed in the testis and ovary, J Biol Chem 277(40) (2002) 37678-84.

[24] D. Schuppan, M. Schmid, R. Somasundaram, R. Ackermann, M. Ruehl, T. Nakamura, E.O. Riecken, Collagens in the liver extracellular matrix bind hepatocyte growth factor, Gastroenterology 114(1) (1998) 139-52.

[25] A.K. Lynn, I.V. Yannas, W. Bonfield, Antigenicity and immunogenicity of collagen, J Biomed Mater Res B Appl Biomater 71(2) (2004) 343-54.

[26] M.J. Buehler, Nanomechanics of collagen fibrils under varying cross-link densities: atomistic and continuum studies, J Mech Behav Biomed Mater 1(1) (2008) 59-67.

[27] F.O. Schmitt, J. Gross, J.H. Highberger, Tropocollagen and the properties of fibrous collagen, Exp Cell Res (Suppl 3) (1955) 326-34.

[28] M.D. Shoulders, R.T. Raines, Collagen structure and stability, Annu Rev Biochem 78 (2009) 929-58.

[29] D.J. Hulmes, A. Miller, Molecular packing in collagen, Nature 293(5829) (1981) 239-4.

[30] K. Gelse, Collagens—structure, function, and biosynthesis, Advanced Drug Delivery Reviews 55(12) (2003) 1531-1546.

[31] D.R. Eyre, M.A. Weis, J.J. Wu, Advances in collagen cross-link analysis, Methods 45(1) (2008) 65-74.

[32] K.E. Kadler, D.F. Holmes, J.A. Trotter, J.A. Chapman, Collagen fibril formation, Biochem J 316 (Pt 1) (1996) 1-11.

[33] M. Mendler, S.G. Eich-Bender, L. Vaughan, K.H. Winterhalter, P. Bruckner, Cartilage contains mixed fibrils of collagen types II, IX, and XI, J Cell Biol 108(1) (1989) 191-7.

[34] D.A. Parry, A.S. Craig, G.R. Barnes, Tendon and ligament from the horse: an ultrastructural study of collagen fibrils and elastic fibres as a function of age, Proc R Soc Lond B Biol Sci 203(1152) (1978) 293-303.

[35] D.A. Parry, G.R. Barnes, A.S. Craig, A comparison of the size distribution of collagen fibrils in connective tissues as a function of age and a possible relation between fibril size distribution and mechanical properties, Proc R Soc Lond B Biol Sci 203(1152) (1978) 305-21.

[36] I.V. Yannas, D.A. Olson, Linear relaxation analysis of the mechanochemical transformation of collagen fibers, Biopolymers 11(4) (1972) 899-912.

[37] O. Tomkins, H.J. Garzozi, Collagen cross-linking: Strengthening the unstable cornea, Clin Ophthalmol 2(4) (2008) 863-7.

[38] Y.P. Kato, D.L. Christiansen, R.A. Hahn, S.J. Shieh, J.D. Goldstein, F.H. Silver, Mechanical properties of collagen fibres: a comparison of reconstituted and rat tail tendon fibres, Biomaterials 10(1) (1989) 38-42.

[39] G. Lai, Y. Li, G. Li, Effect of concentration and temperature on the rheological behavior of collagen solution, International Journal of Biological Macromolecules 42(3) (2008) 285-291.

[40] G.Y. Li, Z.K. Zhang, B. Shi, Physicochemical properties of collagen, gelatin and collagen hydrolysate derived from bovine limed split wastes, J Soc Leath Tech Ch 90(1) (2006) 23-28.

[41] S.T. Nillesen, P.J. Geutjes, R. Wismans, J. Schalkwijk, W.F. Daamen, T.H. van Kuppevelt, Increased angiogenesis and blood vessel maturation in acellular collagen-heparin scaffolds containing both FGF2 and VEGF, Biomaterials 28(6) (2007) 1123-31.

[42] H.R. Elden, Rate of swelling of collagen, Science 128(3339) (1958) 1624-5.

[43] Q. Zhang, L. Liu, H. Zhou, X. Wu, K.D. Yao, pH-responsive swelling behavior of collagen complex materials, Artif Cells Blood Substit Immobil Biotechnol 28(3) (2000) 255-62.

[44] P. Geutjes, L. Roelofs, H. Hoogenkamp, M. Walraven, B. Kortmann, R. de Gier, F. Farag, D. Tiemessen, M. Sloff, E. Oosterwijk, T. van Kuppevelt, W. Daamen, W. Feitz, Tissue engineered tubular construct for urinary diversion in a preclinical porcine model, The Journal of Urology 188(2) (2012) 653-60.

[45] L. Roelofs, A. Eggink, T. van Kuppevelt, R. Wijnen, P. van Den Berg, J. Crevels, N. Hosper, M. van Luyn, P. Geutjes, W. Feitz, Coverage of Fetal Skin Defects With a Collagen Biomatrix Loaded With Growth Factors in a Sheep Model, Journal of Pediatric Urology 5 (2009) S19-S20.

[46] D.W. Bannister, A.B. Burns, Pepsin treatment of avian skin collagen. Effects on solubility, subunit composition and aggregation properties, Biochem J 129(3) (1972) 677-81.

[47] K. Wolf, S. Alexander, V. Schacht, L.M. Coussens, U.H. von Andrian, J. van Rheenen, E. Deryugina, P. Friedl, Collagen-based cell migration models in vitro and in vivo, Seminars in Cell & Developmental Biology 20(8) (2009) 931-41.

[48] S.H. Nezhadi, P.F. Choong, F. Lotfipour, C.R. Dass, Gelatin-based delivery systems for cancer gene therapy, J Drug Target 17(10) (2009) 731-8.

[49] M.C. Gomez-Guillen, J. Turnay, M.D. Fernandez-Diaz, N. Ulmo, M.A. Lizarbe, P. Montero, Structural and physical properties of gelatin extracted from different marine species: a comparative study, Food Hydrocolloids 16(1) (2002) 25-34.

[50] M. Djabourov, J.P. Lechaire, F. Gaill, Structure and Rheology of Gelatin and Collagen Gels, Biorheology 30(3-4) (1993) 191-205.

[51] M.C. Gomez-Guillen, B. Gimenez, M.E. Lopez-Caballero, M.P. Montero, Functional and bioactive properties of collagen and gelatin from alternative sources: A review, Food Hydrocolloids 25(8) (2011) 1813-1827.

[52] A.A. Dongargaonkar, G.L. Bowlin, H. Yang, Electrospun blends of gelatin and gelatin-dendrimer conjugates as a wound-dressing and drug-delivery platform, Biomacromolecules 14(11) (2013) 4038-45.

[53] A.O. Elzoghby, Gelatin-based nanoparticles as drug and gene delivery systems: reviewing three decades of research, J Control Release 172(3) (2013) 1075-91.

[54] M. Santoro, A.M. Tatara, A.G. Mikos, Gelatin carriers for drug and cell delivery in tissue engineering, J Control Release (2014).

[55] D.O. Thomas-Rueddel, V. Vlasakov, K. Reinhart, R. Jaeschke, H. Rueddel, R. Hutagalung, A. Stacke, C.S. Hartog, Safety of gelatin for volume resuscitation--a systematic review and meta-analysis, Intensive Care Med 38(7) (2012) 1134-42.

[56] B.D. Kevadiya, S. Rajkumar, H.C. Bajaj, S.S. Chettiar, K. Gosai, H. Brahmbhatt, A.S. Bhatt, Y.K. Barvaliya, G.S. Dave, R.K. Kothari, Biodegradable gelatin-ciprofloxacin-montmorillonite composite hydrogels for controlled drug release and wound dressing application, Colloids Surf B Biointerfaces 122C (2014) 175-183.

[57] B. Liu, P. Lin, Y. Shen, Y. Dong, Porous bioceramics reinforced by coating gelatin, J Mater Sci Mater Med 19(3) (2008) 1203-7.

[58] S. Bhat, A. Kumar, Cell proliferation on three-dimensional chitosan-agarose-gelatin cryogel scaffolds for tissue engineering applications, Journal of Bioscience and Bioengineering 114(6) (2012) 663-70.

[59] L. Moller, A. Krause, J. Dahlmann, I. Gruh, A. Kirschning, G. Drager, Preparation and evaluation of hydrogel-composites from methacrylated hyaluronic acid, alginate, and gelatin for tissue engineering, Int J Artif Organs 34(2) (2011) 93-102.

[60] S.M. Mithieux, A.S. Weiss, Elastin, Adv Protein Chem 70 (2005) 437-61.

[61] J. Rosenbloom, W.R. Abrams, R. Mecham, Extracellular matrix 4: the elastic fiber, FASEB journal : official publication of the Federation of American Societies for Experimental Biology 7(13) (1993) 1208-18.

[62] S. Rauscher, R. Pomes, Structural disorder and protein elasticity, Adv Exp Med Biol 725 (2012) 159-83.

[63] D.W. Urry, T.M. Parker, Mechanics of elastin: molecular mechanism of biological elasticity and its relationship to contraction, J Muscle Res Cell Motil 23(5-6) (2002) 543-59.

[64] D. Bedell-Hogan, P. Trackman, W. Abrams, J. Rosenbloom, H. Kagan, Oxidation, cross-linking, and insolubilization of recombinant tropoelastin by purified lysyl oxidase, J Biol Chem 268(14) (1993) 10345-50.

[65] A. Heinz, C.K. Ruttkies, G. Jahreis, C.U. Schrader, K. Wichapong, W. Sippl, F.W. Keeley, R.H. Neubert, C.E. Schmelzer, In vitro cross-linking of elastin peptides and molecular characterization of the resultant biomaterials, Biochim Biophys Acta 1830(4) (2013) 2994-3004.

[66] S.G. Wise, A.S. Weiss, Tropoelastin, Int J Biochem Cell Biol 41(3) (2009) 494-7.

[67] T.J. Broekelmann, B.A. Kozel, H. Ishibashi, C.C. Werneck, F.W. Keeley, L. Zhang, R.P. Mecham, Tropoelastin interacts with cell-surface glycosaminoglycans via its COOH-terminal domain, J Biol Chem 280(49) (2005) 40939-47.

[68] S. Dutoya, A. Verna, F. Lefebvre, M. Rabaud, Elastin-derived protein coating onto poly(ethylene terephthalate). Technical, microstructural and biological studies, Biomaterials 21(15) (2000) 1521-9.

[69] K.A. Woodhouse, P. Klement, V. Chen, M.B. Gorbet, F.W. Keeley, R. Stahl, J.D. Fromstein, C.M. Bellingham, Investigation of recombinant human elastin polypeptides as non-thrombogenic coatings, Biomaterials 25(19) (2004) 4543-53.

[70] W.F. Daamen, S.T. Nillesen, R.G. Wismans, D.P. Reinhardt, T. Hafmans, J.H. Veerkamp, T.H. van Kuppevelt, A biomaterial composed of collagen and solubilized elastin enhances angiogenesis and elastic fiber formation without calcification, Tissue Eng Part A 14(3) (2008) 349-60.

[71] V.L. Sales, G.C. Engelmayr, Jr., J.A. Johnson, Jr., J. Gao, Y. Wang, M.S. Sacks, J.E. Mayer, Jr., Protein precoating of elastomeric tissue-engineering scaffolds increased cellularity, enhanced extracellular matrix protein production, and differentially regulated the phenotypes of circulating endothelial progenitor cells, Circulation 116(11 Suppl) (2007) I55-63.

[72] N. Annabi, S.M. Mithieux, E.A. Boughton, A.J. Ruys, A.S. Weiss, F. Dehghani, Synthesis of highly porous crosslinked elastin hydrogels and their interaction with fibroblasts in vitro, Biomaterials 30(27) (2009) 4550-7.

[73] S. Heydarkhan-Hagvall, K. Schenke-Layland, A.P. Dhanasopon, F. Rofail, H. Smith, B.M. Wu, R. Shemin, R.E. Beygui, W.R. MacLellan, Three-dimensional electrospun ECM-based hybrid scaffolds for cardiovascular tissue engineering, Biomaterials 29(19) (2008) 2907-14.

[74] J. Rnjak, S.G. Wise, S.M. Mithieux, A.S. Weiss, Severe burn injuries and the role of elastin in the design of dermal substitutes, Tissue Eng Part B Rev 17(2) (2011) 81-91.

[75] L. Nivison-Smith, J. Rnjak, A.S. Weiss, Synthetic human elastin microfibers: stable cross-linked tropoelastin and cell interactive constructs for tissue engineering applications, Acta Biomaterialia 6(2) (2010) 354-9.

[76] J.F. Almine, D.V. Bax, S.M. Mithieux, L. Nivison-Smith, J. Rnjak, A. Waterhouse, S.G. Wise, A.S. Weiss, Elastin-based materials, Chem Soc Rev 39(9) (2010) 3371-9.

[77] W.F. Daamen, J.H. Veerkamp, J.C. van Hest, T.H. van Kuppevelt, Elastin as a biomaterial for tissue engineering, Biomaterials 28(30) (2007) 4378-98.

[78] B. Sivaraman, C.A. Bashur, A. Ramamurthi, Advances in biomimetic regeneration of elastic matrix structures, Drug Deliv Transl Res 2(5) (2012) 323-350.

[79] F. Rosso, A. Giordano, M. Barbarisi, A. Barbarisi, From cell-ECM interactions to tissue engineering, J Cell Physiol 199(2) (2004) 174-80.

[80] Z. Mostafavi-Pour, J.A. Askari, S.J. Parkinson, P.J. Parker, T.T. Ng, M.J. Humphries, Integrin-specific signaling pathways controlling focal adhesion formation and cell migration, J Cell Biol 161(1) (2003) 155-67.

[81] E. Ruoslahti, M.D. Pierschbacher, Arg-Gly-Asp: a versatile cell recognition signal, Cell 44(4) (1986) 517-8.

[82] P. Friedl, E.B. Brocker, The biology of cell locomotion within three-dimensional extracellular matrix, Cell Mol Life Sci 57(1) (2000) 41-64.

[83] J. Xu, D. Mosher, Fibronectin and other adhesive glycoproteins, in: R.P. Mecham (Ed.), The Extracellular Matrix: an Overview, Springer Berlin Heidelberg2011, pp. 41-75.

[84] D.S. Gould, H. Auchincloss, Jr., Direct and indirect recognition: the role of MHC antigens in graft rejection, Immunol Today 20(2) (1999) 77-82.

[85] Y. Yamada, K. Hozumi, A. Aso, A. Hotta, K. Toma, F. Katagiri, Y. Kikkawa, M. Nomizu, Laminin active peptide/agarose matrices as multifunctional biomaterials for tissue engineering, Biomaterials 33(16) (2012) 4118-25.

[86] M.M. Martino, F. Tortelli, M. Mochizuki, S. Traub, D. Ben-David, G.A. Kuhn, R. Muller, E. Livne, S.A. Eming, J.A. Hubbell, Engineering the growth factor microenvironment with fibronectin domains to promote wound and bone tissue healing, Sci Transl Med 3(100) (2011) 100ra89.

[87] M. Aumailley, L. Bruckner-Tuderman, W.G. Carter, R. Deutzmann, D. Edgar, P. Ekblom, J. Engel, E. Engvall, E. Hohenester, J.C. Jones, H.K. Kleinman, M.P. Marinkovich, G.R. Martin, U. Mayer, G. Meneguzzi, J.H. Miner, K. Miyazaki, M. Patarroyo, M. Paulsson, V. Quaranta, J.R. Sanes, T. Sasaki, K. Sekiguchi, L.M. Sorokin, J.F. Talts, K. Tryggvason, J. Uitto, I. Virtanen, K. von der Mark, U.M. Wewer, Y. Yamada, P.D. Yurchenco, A simplified laminin nomenclature, Matrix Biol 24(5) (2005) 326-32.

[88] Y. Iwamoto, F.A. Robey, J. Graf, M. Sasaki, H.K. Kleinman, Y. Yamada, G.R. Martin, YIGSR, a synthetic laminin pentapeptide, inhibits experimental metastasis formation, Science 238(4830) (1987) 1132-4.

[89] A.M. Wojtowicz, A. Shekaran, M.E. Oest, K.M. Dupont, K.L. Templeman, D.W. Hutmacher, R.E. Guldberg, A.J. Garcia, Coating of biomaterial scaffolds with the collagen-mimetic peptide GFOGER for bone defect repair, Biomaterials 31(9) (2010) 2574-82.

[90] S. Pradhan, C. Zhang, X. Jia, D.D. Carson, R. Witt, M.C. Farach-Carson, Perlecan domain IV peptide stimulates salivary gland cell assembly in vitro, Tissue Eng Part A 15(11) (2009) 3309-20.

[91] R. Timpl, H. Rohde, P.G. Robey, S.I. Rennard, J.M. Foidart, G.R. Martin, Laminin--a glycoprotein from basement membranes, J Biol Chem 254(19) (1979) 9933-7.

[92] J.F. Tarsio, L.A. Reger, L.T. Furcht, Molecular mechanisms in basement membrane complications of diabetes. Alterations in heparin, laminin, and type IV collagen association, Diabetes 37(5) (1988) 532-9.

[93] J. Kruegel, N. Miosge, Basement membrane components are key players in specialized extracellular matrices, Cell Mol Life Sci 67(17) (2010) 2879-95.

[94] A. El-Ghannam, L. Starr, J. Jones, Laminin-5 coating enhances epithelial cell attachment, spreading, and hemidesmosome assembly on Ti-6A1-4V implant material in vitro, Journal of Biomedical Materials Research 41(1) (1998) 30-40.

[95] H. Kosmehl, A. Berndt, D. Katenkamp, Molecular variants of fibronectin and laminin: structure, physiological occurrence and histopathological aspects, Virchows Arch 429(6) (1996) 311-22.

[96] R. Pankov, K.M. Yamada, Fibronectin at a glance, J Cell Sci 115(Pt 20) (2002) 3861-3.

[97] M.W. Mosesson, K.R. Siebenlist, D.A. Meh, The structure and biological features of fibrinogen and fibrin, Ann N Y Acad Sci 936 (2001) 11-30.

[98] J.W. Weisel, C.H. Dempfle, Fibrinogen Structure and Function, in: V.J. Marder, W.C. Aird, J.G. Bennett, S. Schulman, G.C. White (Eds.), Hemostasis and Thrombosis: Basic Principles and Clinial Practice, Lippincot Williams & Wilkins, Philadelphia, 2012.

[99] C. Longstaff, C. Thelwell, S.C. Williams, M.M. Silva, L. Szabo, K. Kolev, The interplay between tissue plasminogen activator domains and fibrin structures in the regulation of fibrinolysis: kinetic and microscopic studies, Blood 117(2) (2011) 661-8.

[100] S. Pradhan, M.C. Farach-Carson, Mining the extracellular matrix for tissue engineering applications, Regen Med 5(6) (2010) 961-70.

[101] R. Glaum, M. Wiedmann-Al-Ahmad, U. Huebner, R. Schmelzeisen, Tissue engineering of composite grafts: Cocultivation of human oral keratinocytes and human osteoblast-like cells on laminin-coated polycarbonate membranes and equine collagen membranes under different culture conditions, J Biomed Mater Res A 93(2) (2010) 704-15.

[102] R. Junka, C.M. Valmikinathan, D.M. Kalyon, X. Yu, Laminin Functionalized Biomimetic Nanofibers For Nerve Tissue Engineering, J Biomater Tissue Eng 3(4) (2013) 494-502.

[103] S. Suri, C.E. Schmidt, Cell-laden hydrogel constructs of hyaluronic acid, collagen, and laminin for neural tissue engineering, Tissue Eng Part A 16(5) (2010) 1703-16.

[104] S.E. Stabenfeldt, A.J. Garcia, M.C. LaPlaca, Thermoreversible laminin-functionalized hydrogel for neural tissue engineering, J Biomed Mater Res A 77(4) (2006) 718-25.

[105] C. Deister, S. Aljabari, C.E. Schmidt, Effects of collagen 1, fibronectin, laminin and hyaluronic acid concentration in multi-component gels on neurite extension, J Biomater Sci Polym Ed 18(8) (2007) 983-97.

[106] A.T. Francisco, P.Y. Hwang, C.G. Jeong, L. Jing, J. Chen, L.A. Setton, Photocrosslinkable laminin-functionalized polyethylene glycol hydrogel for intervertebral disc regeneration, Acta Biomaterialia 10(3) (2014) 1102-11.

[107] K. McEwan, D.T. Padavan, C. Ellis, J.E. McBane, B. Vulesevic, G.S. Korbutt, E.J. Suuronen, Collagen-chitosan-laminin hydrogels for the delivery of insulin-producing tissue, Journal of Tissue Engineering and Regenerative Medicine (2013).

[108] J.B. Phillips, V.R. King, Z. Ward, R.A. Porter, J.V. Priestley, R.A. Brown, Fluid shear in viscous fibronectin gels allows aggregation of fibrous materials for CNS tissue engineering, Biomaterials 25(14) (2004) 2769-79.

[109] S. Grigorescu, M. Hindie, E. Axente, F. Carreiras, K. Anselme, J. Werckmann, I.N. Mihailescu, O. Gallet, Fabrication of functional fibronectin patterns by nanosecond excimer laser direct write for tissue engineering applications, J Mater Sci Mater Med 24(7) (2013) 1809-21.

[110] G. Dubey, K. Mequanint, Conjugation of fibronectin onto three-dimensional porous scaffolds for vascular tissue engineering applications, Acta Biomaterialia 7(3) (2011) 1114-25.

[111] S. Ravi, J.M. Caves, A.W. Martinez, C.A. Haller, E.L. Chaikof, Incorporation of fibronectin to enhance cytocompatibility in multilayer elastin-like protein scaffolds for tissue engineering, J Biomed Mater Res A 101(7) (2013) 1915-25.

[112] S. Underwood, A. Afoke, R.A. Brown, A.J. MacLeod, P.A. Shamlou, P. Dunnill, Wet extrusion of fibronectin-fibrinogen cables for application in tissue engineering, Biotechnol Bioeng 73(4) (2001) 295-305.

[113] T. Bielecki, D.M. Dohan Ehrenfest, Platelet-rich plasma (PRP) and Platelet-Rich Fibrin (PRF): surgical adjuvants, preparations for in situ regenerative medicine and tools for tissue engineering, Curr Pharm Biotechnol 13(7) (2012) 1121-30.

[114] M. Dietrich, J. Heselhaus, J. Wozniak, S. Weinandy, P. Mela, B. Tschoeke, T. Schmitz-Rode, S. Jockenhoevel, Fibrin-based tissue engineering: comparison of different methods of autologous fibrinogen isolation, Tissue Eng Part C Methods 19(3) (2013) 216-26.

[115] I. Frisman, Y. Shachaf, D. Seliktar, H. Bianco-Peled, Stimulus-responsive hydrogels made from biosynthetic fibrinogen conjugates for tissue engineering: structural characterization, Langmuir 27(11) (2011) 6977-86.

[116] S.A. Sell, M.P. Francis, K. Garg, M.J. McClure, D.G. Simpson, G.L. Bowlin, Cross-linking methods of electrospun fibrinogen scaffolds for tissue engineering applications, Biomed Mater 3(4) (2008) 045001.

[117] C. He, X. Xu, F. Zhang, L. Cao, W. Feng, H. Wang, X. Mo, Fabrication of fibrinogen/P(LLA-CL) hybrid nanofibrous scaffold for potential soft tissue engineering applications, J Biomed Mater Res A 97(3) (2011) 339-47.

[118] M.P. Linnes, B.D. Ratner, C.M. Giachelli, A fibrinogen-based precision microporous scaffold for tissue engineering, Biomaterials 28(35) (2007) 5298-306.

[119] S.W. Cho, S.S. Kim, J.W. Rhie, H.M. Cho, C.Y. Choi, B.S. Kim, Engineering of volume-stable adipose tissues, Biomaterials 26(17) (2005) 3577-85.

[120] H. Zhou, H.H. Xu, The fast release of stem cells from alginate-fibrin microbeads in injectable scaffolds for bone tissue engineering, Biomaterials 32(30) (2011) 7503-13.

[121] M. Weber, E. Heta, R. Moreira, V.N. Gesche, T. Schermer, J. Frese, S. Jockenhoevel, P. Mela, Tissue-engineered fibrin-based heart valve with a tubular leaflet design, Tissue Eng Part C Methods 20(4) (2014) 265-75.

[122] D. Eyrich, F. Brandl, B. Appel, H. Wiese, G. Maier, M. Wenzel, R. Staudenmaier, A. Goepferich, T. Blunk, Long-term stable fibrin gels for cartilage engineering, Biomaterials 28(1) (2007) 55-65.

[123] R.L. Page, C. Malcuit, L. Vilner, I. Vojtic, S. Shaw, E. Hedblom, J. Hu, G.D. Pins, M.W. Rolle, T. Dominko, Restoration of skeletal muscle defects with adult human cells delivered on fibrin microthreads, Tissue Eng Part A 17(21-22) (2011) 2629-40.

[124] S.M. Willerth, K.J. Arendas, D.I. Gottlieb, S.E. Sakiyama-Elbert, Optimization of fibrin scaffolds for differentiation of murine embryonic stem cells into neural lineage cells, Biomaterials 27(36) (2006) 5990-6003.

[125] M. Alaminos, M. Del Carmen Sanchez-Quevedo, J.I. Munoz-Avila, D. Serrano, S. Medialdea, I. Carreras, A. Campos, Construction of a complete rabbit cornea substitute using a fibrin-agarose scaffold, Invest Ophthalmol Vis Sci 47(8) (2006) 3311-7.

[126] C.G. Cornelissen, M. Dietrich, S. Kruger, J. Spillner, T. Schmitz-Rode, S. Jockenhoevel, Fibrin gel as alternative scaffold for respiratory tissue engineering, Ann Biomed Eng 40(3) (2012) 679-87.

[127] V. Carriel, I. Garzon, J.M. Jimenez, A.C. Oliveira, S. Arias-Santiago, A. Campos, M.C. Sanchez-Quevedo, M. Alaminos, Epithelial and stromal developmental patterns in a novel substitute of the human skin generated with fibrin-agarose biomaterials, Cells Tissues Organs 196(1) (2012) 1-12.

[128] S. Hankemeier, M. van Griensven, M. Ezechieli, T. Barkhausen, M. Austin, M. Jagodzinski, R. Meller, U. Bosch, C. Krettek, J. Zeichen, Tissue engineering of tendons and ligaments by human bone marrow stromal cells in a liquid fibrin matrix in immunodeficient rats: results of a histologic study, Arch Orthop Trauma Surg 127(9) (2007) 815-21.

[129] J.Z. Paxton, U.N. Wudebwe, A. Wang, D. Woods, L.M. Grover, Monitoring sinew contraction during formation of tissue-engineered fibrin-based ligament constructs, Tissue Eng Part A 18(15-16) (2012) 1596-607.

[130] A. Lesman, J. Koffler, R. Atlas, Y.J. Blinder, Z. Kam, S. Levenberg, Engineering vessel-like networks within multicellular fibrin-based constructs, Biomaterials 32(31) (2011) 7856-69.

[131] P. de la Puente, D. Ludena, Cell culture in autologous fibrin scaffolds for applications in tissue engineering, Exp Cell Res 322(1) (2014) 1-11.

[132] M.C. Barsotti, F. Felice, A. Balbarini, R. Di Stefano, Fibrin as a scaffold for cardiac tissue engineering, Biotechnol Appl Biochem 58(5) (2011) 301-10.

[133] A.V. Kajava, J.M. Squire, D.A. Parry, Beta-structures in fibrous proteins, Adv Protein Chem 73 (2006) 1-15.

[134] E. Mitscherlich, Treatments of strictures by horsehair bougies, American Journal of the Medical Sciences 121(1) (1871) 275.

[135] R.E. Kravetz, Horse hair sutures, Am J Gastroenterol 98(3) (2003) 691.

[136] X. Pan, R.P. Hobbs, P.A. Coulombe, The expanding significance of keratin intermediate filaments in normal and diseased epithelia, Curr Opin Cell Biol 25(1) (2013) 47-56.

[137] R.C. Marshall, D.F. Orwin, J.M. Gillespie, Structure and biochemistry of mammalian hard keratin, Electron Microsc Rev 4(1) (1991) 47-83.

[138] A. Kolsch, R. Windoffer, T. Wurflinger, T. Aach, R.E. Leube, The keratin-filament cycle of assembly and disassembly, J Cell Sci 123(Pt 13) (2010) 2266-72.

[139] B. Srinivasan, R. Kumar, K. Shanmugam, U.T. Sivagnam, N.P. Reddy, P.K. Sehgal, Porous keratin scaffold-promising biomaterial for tissue engineering and drug delivery, J Biomed Mater Res B Appl Biomater 92(1) (2010) 5-12.

[140] W.R. Dong, B.L. Zhao, Y.Q. Xiao, X.X. Qiu, Y.H. Chen, Z.Z. Zou, Toxicity evaluation of chicken calamus keratin conduit as a tissue-engineering scaffold biomaterial, Nan Fang Yi Ke Da Xue Xue Bao 27(7) (2007) 931-5.

[141] D.F. Qiao, Y.M. Lu, W.Y. Fu, Y.J. Piao, Degradation of human hair keratin scaffold implanted for repairing injured skeletal muscles, Di Yi Jun Yi Da Xue Xue Bao 22(10) (2002) 902-4.

[142] P.S. Hill, P.J. Apel, J. Barnwell, T. Smith, L.A. Koman, A. Atala, M. Van Dyke, Repair of peripheral nerve defects in rabbits using keratin hydrogel scaffolds, Tissue Eng Part A 17(11-12) (2011) 1499-505.

[143] A. Tachibana, Y. Furuta, H. Takeshima, T. Tanabe, K. Yamauchi, Fabrication of wool keratin sponge scaffolds for long-term cell cultivation, J Biotechnol 93(2) (2002) 165-70.

[144] S. Reichl, Films based on human hair keratin as substrates for cell culture and tissue engineering, Biomaterials 30(36) (2009) 6854-66.

[145] L.A. Pace, J.F. Plate, S. Mannava, J.C. Barnwell, L.A. Koman, Z. Li, T.L. Smith, M. Van Dyke, A human hair keratin hydrogel scaffold enhances median nerve regeneration in nonhuman primates: an electrophysiological and histological study, Tissue Eng Part A 20(3-4) (2014) 507-17.

[146] P.J. Apel, J.P. Garrett, P. Sierpinski, J. Ma, A. Atala, T.L. Smith, L.A. Koman, M.E. Van Dyke, Peripheral nerve regeneration using a keratin-based scaffold: long-term functional and histological outcomes in a mouse model, J Hand Surg Am 33(9) (2008) 1541-7.

[147] S. Saravanan, D.K. Sameera, A. Moorthi, N. Selvamurugan, Chitosan scaffolds containing chicken feather keratin nanoparticles for bone tissue engineering, International Journal of Biological Macromolecules 62 (2013) 481-6.

[148] A. Tachibana, S. Kaneko, T. Tanabe, K. Yamauchi, Rapid fabrication of keratin-hydroxyapatite hybrid sponges toward osteoblast cultivation and differentiation, Biomaterials 26(3) (2005) 297-302.

[149] J.H. Yoon, J. Halper, Tendon proteoglycans: biochemistry and function, J Musculoskelet Neuronal Interact 5(1) (2005) 22-34.

[150] J.E. Scott, Structure and function in extracellular matrices depend on interactions between anionic glycosaminoglycans, Pathol Biol (Paris) 49(4) (2001) 284-9.

[151] C.R. Parish, The role of heparan sulphate in inflammation, Nat Rev Immunol 6(9) (2006) 633-43.

[152] C.J. Jones, S. Beni, J.F. Limtiaco, D.J. Langeslay, C.K. Larive, Heparin characterization: challenges and solutions, Annu Rev Anal Chem (Palo Alto Calif) 4 (2011) 439-65.

[153] K. Sugahara, T. Mikami, T. Uyama, S. Mizuguchi, K. Nomura, H. Kitagawa, Recent advances in the structural biology of chondroitin sulfate and dermatan sulfate, Curr Opin Struct Biol 13(5) (2003) 612-20.

[154] J.L. Funderburgh, Keratan sulfate: structure, biosynthesis, and function, Glycobiology 10(10) (2000) 951-8.

[155] D.D. Allison, K.J. Grande-Allen, Review. Hyaluronan: a powerful tissue engineering tool, Tissue Engineering 12(8) (2006) 2131-40.

[156] I.I. Fuki, R.V. Iozzo, K.J. Williams, Perlecan heparan sulfate proteoglycan. A novel receptor that mediates a distinct pathway for ligand catabolism, J Biol Chem 275(40) (2000) 31554.

[157] C. Kiani, L. Chen, Y.J. Wu, A.J. Yee, B.B. Yang, Structure and function of aggrecan, Cell Res 12(1) (2002) 19-32.

[158] T.N. Wight, Versican: a versatile extracellular matrix proteoglycan in cell biology, Curr Opin Cell Biol 14(5) (2002) 617-23.

[159] Y. Yamaguchi, Lecticans: organizers of the brain extracellular matrix, Cell Mol Life Sci 57(2) (2000) 276-89.

[160] U. Rauch, K. Feng, X.H. Zhou, Neurocan: a brain chondroitin sulfate proteoglycan, Cell Mol Life Sci 58(12-13) (2001) 1842-56.

[161] M. Kolb, P.J. Margetts, P.J. Sime, J. Gauldie, Proteoglycans decorin and biglycan differentially modulate TGF-beta-mediated fibrotic responses in the lung, Am J Physiol Lung Cell Mol Physiol 280(6) (2001) L1327-34.

[162] E. Mikaelsson, A.H. Danesh-Manesh, A. Luppert, M. Jeddi-Tehrani, M.R. Rezvany, R.A. Sharifian, R. Safaie, A. Roohi, A. Osterborg, F. Shokri, H. Mellstedt, H. Rabbani, Fibromodulin, an extracellular matrix protein: characterization of its unique gene and protein expression in B-cell chronic lymphocytic leukemia and mantle cell lymphoma, Blood 105(12) (2005) 4828-35.

[163] M. Ly, F.E. Leach, 3rd, T.N. Laremore, T. Toida, I.J. Amster, R.J. Linhardt, The proteoglycan bikunin has a defined sequence, Nat Chem Biol 7(11) (2011) 827-33.

[164] K. Elenius, M. Jalkanen, Function of the syndecans--a family of cell surface proteoglycans, J Cell Sci 107 (Pt 11) (1994) 2975-82.

[165] E.C. Carlson, C.Y. Liu, T. Chikama, Y. Hayashi, C.W. Kao, D.E. Birk, J.L. Funderburgh, J.V. Jester, W.W. Kao, Keratocan, a cornea-specific keratan sulfate proteoglycan, is regulated by lumican, J Biol Chem 280(27) (2005) 25541-7.

[166] P. Lu, K. Takai, V.M. Weaver, Z. Werb, Extracellular matrix degradation and remodeling in development and disease, Cold Spring Harb Perspect Biol 3(12) (2011).

[167] G. Lammers, E.M. van de Westerlo, E.M. Versteeg, T.H. van Kuppevelt, W.F. Daamen, A Comparison of Seven Methods to Analyze Heparin in Biomaterials: Quantification, Location, and Anticoagulant Activity, Tissue Eng Part C Methods (2011).

[168] R.J. Linhardt, S.A. Ampofo, J. Fareed, D. Hoppensteadt, J.B. Mulliken, J. Folkman, Isolation and characterization of human heparin, Biochemistry 31(49) (1992) 12441-5.

[169] J.D. Esko, K. Kimata, U. Lindahl, Proteoglycans and Sulfated Glycosaminoglycans, in: A. Varki, R.D. Cummings, J.D. Esko, H.H. Freeze, P. Stanley, C.R. Bertozzi, G.W. Hart, M.E. Etzler (Eds.), Essentials of Glycobiology, Cold Spring Harbor (NY), 2009.

[170] M. Fath, V. VanderNoot, I. Kilpelainen, T. Kinnunen, H. Rauvala, R.J. Linhardt, Interaction of soluble and surface-bound heparin binding growth-associated molecule with heparin, FEBS Lett 454(1-2) (1999) 105-8.

[171] J.T. Gallagher, A. Walker, Molecular distinctions between heparan sulphate and heparin. Analysis of sulphation patterns indicates that heparan sulphate and heparin are separate families of N-sulphated polysaccharides, Biochem J 230(3) (1985) 665-74.

[172] J.D. Esko, S.B. Selleck, Order out of chaos: assembly of ligand binding sites in heparan sulfate, Annu Rev Biochem 71 (2002) 435-71.

[173] J.S. Pieper, P.B. van Wachem, M.J.A. van Luyn, L.A. Brouwer, T. Hafmans, J.H. Veerkamp, T.H. van Kuppevelt, Attachment of glycosaminoglycans to collagenous matrices modulates the tissue response in rats, Biomaterials 21(16) (2000) 1689-99.

[174] R. Sasisekharan, G. Venkataraman, Heparin and heparan sulfate: biosynthesis, structure and function, Curr Opin Chem Biol 4(6) (2000) 626-31.

[175] U. Lindahl, M. Kusche-Gullberg, L. Kjellen, Regulated diversity of heparan sulfate, J Biol Chem 273(39) (1998) 24979-82.

[176] S.A. Baeurle, M.G. Kiselev, E.S. Makarova, E.A. Nogovitsin, Effect of the counterion behavior on the frictional-compressive properties of chondroitin sulfate solutions, Polymer 50(7) (2009) 1805-1813.

[177] C. Malavaki, S. Mizumoto, N. Karamanos, K. Sugahara, Recent advances in the structural study of functional chondroitin sulfate and dermatan sulfate in health and disease, Connect Tissue Res 49(3) (2008) 133-9.

[178] K. Sugahara, T. Mikami, Chondroitin/dermatan sulfate in the central nervous system, Curr Opin Struct Biol 17(5) (2007) 536-45.

[179] T.E. McAlindon, M.P. LaValley, J.P. Gulin, D.T. Felson, Glucosamine and chondroitin for treatment of osteoarthritis: a systematic quality assessment and meta-analysis, Jama 283(11) (2000) 1469-75.

[180] J.L.C. van Susante, J. Pieper, P. Buma, T.H. van Kuppevelt, H. van Beuningen, P.M. van Der Kraan, J.H. Veerkamp, W.B. van den Berg, R.P.H. Veth, Linkage of chondroitin-sulfate to type I collagen scaffolds stimulates the bioactivity of seeded chondrocytes in vitro, Biomaterials 22(17) (2001) 2359-69.

[181] R.A. Muzzarelli, F. Greco, A. Busilacchi, V. Sollazzo, A. Gigante, Chitosan, hyaluronan and chondroitin sulfate in tissue engineering for cartilage regeneration: a review, Carbohydrate Polymers 89(3) (2012) 723-39.

[182] S.S. Deepa, Y. Umehara, S. Higashiyama, N. Itoh, K. Sugahara, Specific molecular interactions of oversulfated chondroitin sulfate E with various heparin-binding growth factors. Implications as a physiological binding partner in the brain and other tissues, J Biol Chem 277(46) (2002) 43707-16.

[183] T.J. Keane, S.F. Badylak, Biomaterials for tissue engineering applications, Seminars in Pediatric Surgery 23(3) (2014) 112-8.

[184] J.M. Trowbridge, J.A. Rudisill, D. Ron, R.L. Gallo, Dermatan sulfate binds and potentiates activity of keratinocyte growth factor (FGF-7), J Biol Chem 277(45) (2002) 42815-20.

[185] S.F. Penc, B. Pomahac, T. Winkler, R.A. Dorschner, E. Eriksson, M. Herndon, R.L. Gallo, Dermatan sulfate released after injury is a potent promoter of fibroblast growth factor-2 function, J Biol Chem 273(43) (1998) 28116-21.

[186] K. Nikolovska, J.K. Renke, O. Jungmann, K. Grobe, R.V. Iozzo, A.D. Zamfir, D.G. Seidler, A decorin-deficient matrix affects skin chondroitin/dermatan sulfate levels and keratinocyte function, Matrix Biol 35 (2014) 91-102.

[187] J.M. Trowbridge, R.L. Gallo, Dermatan sulfate: new functions from an old glycosaminoglycan, Glycobiology 12(9) (2002) 117R-25R.

[188] J.M. Dickenson, H.G. Morris, I.A. Nieduszynski, T.N. Huckerby, Skeletal keratan sulfate chain molecular weight calibration by high-performance gel-permeation chromatography, Anal Biochem 190(2) (1990) 271-5.

[189] J.R. Hassell, C. Cintron, C. Kublin, D.A. Newsome, Proteoglycan changes during restoration of transparency in corneal scars, Arch Biochem Biophys 222(2) (1983) 362-9.

[190] J.I. Kuiper, J.H. Verbeek, M.H. Frings-Dresen, A.J. Ikkink, Keratan sulfate as a potential biomarker of loading of the intervertebral disc, Spine (Phila Pa 1976) 23(6) (1998) 657-63.

[191] R.D. Young, E.C. Gealy, M. Liles, B. Caterson, J.R. Ralphs, A.J. Quantock, Keratan sulfate glycosaminoglycan and the association with collagen fibrils in rudimentary lamellae in the developing avian cornea, Invest Ophthalmol Vis Sci 48(7) (2007) 3083-8.

[192] J.A. Greenwood, J.E. Murphy-Ullrich, Signaling of de-adhesion in cellular regulation and motility, Microsc Res Tech 43(5) (1998) 420-32.

[193] J.L. Funderburgh, R.R. Mitschler, M.L. Funderburgh, M.R. Roth, S.K. Chapes, G.W. Conrad, Macrophage receptors for lumican. A corneal keratan sulfate proteoglycan, Invest Ophthalmol Vis Sci 38(6) (1997) 1159-67.

[194] S. Sharma, A. Panitch, C.P. Neu, Incorporation of an aggrecan mimic prevents proteolytic degradation of anisotropic cartilage analogs, Acta Biomaterialia 9(1) (2013) 4618-25.

[195] A.J. Quantock, R.D. Young, T.O. Akama, Structural and biochemical aspects of keratan sulphate in the cornea, Cell Mol Life Sci 67(6) (2010) 891-906.

[196] J.L. Funderburgh, M.L. Funderburgh, M.M. Mann, G.W. Conrad, Physical and biological properties of keratan sulphate proteoglycan, Biochem Soc Trans 19(4) (1991) 871-6.

[197] J.R. Fraser, T.C. Laurent, U.B. Laurent, Hyaluronan: its nature, distribution, functions and turnover, J Intern Med 242(1) (1997) 27-33.

[198] T.C. Laurent, J.R. Fraser, Hyaluronan, FASEB journal : official publication of the Federation of American Societies for Experimental Biology 6(7) (1992) 2397-404.

[199] W. Puhl, P. Scharf, Intra-articular hyaluronan treatment for osteoarthritis, Ann Rheum Dis 56(7) (1997) 441.

[200] A. Darr, A. Calabro, Synthesis and characterization of tyramine-based hyaluronan hydrogels, J Mater Sci Mater Med 20(1) (2009) 33-44.

[201] S. Gerecht, J.A. Burdick, L.S. Ferreira, S.A. Townsend, R. Langer, G. Vunjak-Novakovic, Hyaluronic acid hydrogel for controlled self-renewal and differentiation of human embryonic stem cells, Proc Natl Acad Sci U S A 104(27) (2007) 11298-303.

[202] X.Z. Shu, K. Ghosh, Y. Liu, F.S. Palumbo, Y. Luo, R.A. Clark, G.D. Prestwich, Attachment and spreading of fibroblasts on an RGD peptide-modified injectable hyaluronan hydrogel, J Biomed Mater Res A 68(2) (2004) 365-75.

[203] L. Bacakova, K. Novotna, M. Parizek, Polysaccharides as cell carriers for tissue engineering: the use of cellulose in vascular wall reconstruction, Physiol Res 63 Suppl 1 (2014) S29-47.

[204] F. Vollrath, D.P. Knight, Liquid crystalline spinning of spider silk, Nature 410(6828) (2001) 541-8.

[205] F. Vollrath, Biology of spider silk, International Journal of Biological Macromolecules 24(2-3) (1999) 81-8.

[206] C. Wong Po Foo, D.L. Kaplan, Genetic engineering of fibrous proteins: spider dragline silk and collagen, Advanced Drug Delivery Reviews 54(8) (2002) 1131-43.

[207] A. Rising, H. Nimmervoll, S. Grip, A. Fernandez-Arias, E. Storckenfeldt, D.P. Knight, F. Vollrath, W. Engstrom, Spider silk proteins--mechanical property and gene sequence, Zoolog Sci 22(3) (2005) 273-81.

[208] C. Vepari, D.L. Kaplan, Silk as a Biomaterial, Prog Polym Sci 32(8-9) (2007) 991-1007.

[209] Y. Wang, H.J. Kim, G. Vunjak-Novakovic, D.L. Kaplan, Stem cell-based tissue engineering with silk biomaterials, Biomaterials 27(36) (2006) 6064-82.

[210] D.G. Harkin, K.A. George, P.W. Madden, I.R. Schwab, D.W. Hutmacher, T.V. Chirila, Silk fibroin in ocular tissue reconstruction, Biomaterials 32(10) (2011) 2445-58.

[211] X. Wang, H.J. Kim, P. Xu, A. Matsumoto, D.L. Kaplan, Biomaterial coatings by stepwise deposition of silk fibroin, Langmuir 21(24) (2005) 11335-41.

[212] U.J. Kim, J. Park, C. Li, H.J. Jin, R. Valluzzi, D.L. Kaplan, Structure and properties of silk hydrogels, Biomacromolecules 5(3) (2004) 786-92.

[213] A. Schneider, X.Y. Wang, D.L. Kaplan, J.A. Garlick, C. Egles, Biofunctionalized electrospun silk mats as a topical bioactive dressing for accelerated wound healing, Acta Biomaterialia 5(7) (2009) 2570-8.

[214] I. Dal Pra, G. Freddi, J. Minic, A. Chiarini, U. Armato, De novo engineering of reticular connective tissue in vivo by silk fibroin nonwoven materials, Biomaterials 26(14) (2005) 1987-99.

[215] U.J. Kim, J. Park, H.J. Kim, M. Wada, D.L. Kaplan, Three-dimensional aqueous-derived biomaterial scaffolds from silk fibroin, Biomaterials 26(15) (2005) 2775-85.

[216] R. Nazarov, H.J. Jin, D.L. Kaplan, Porous 3-D scaffolds from regenerated silk fibroin, Biomacromolecules 5(3) (2004) 718-26.

[217] Y. Tamada, New process to form a silk fibroin porous 3-D structure, Biomacromolecules 6(6) (2005) 3100-6.

[218] G.H. Altman, F. Diaz, C. Jakuba, T. Calabro, R.L. Horan, J. Chen, H. Lu, J. Richmond, D.L. Kaplan, Silk-based biomaterials, Biomaterials 24(3) (2003) 401-16.

[219] K.Y. Lee, D.J. Mooney, Alginate: properties and biomedical applications, Prog Polym Sci 37(1) (2012) 106-126.

[220] M. Steigedal, H. Sletta, S. Moreno, M. Maerk, B.E. Christensen, T. Bjerkan, T.E. Ellingsen, G. Espin, H. Ertesvag, S. Valla, The Azotobacter vinelandii AlgE mannuronan C-5-epimerase family is essential for the in vivo control of alginate monomer composition and for functional cyst formation, Environ Microbiol 10(7) (2008) 1760-70.

[221] M. Borgogna, G. Skjak-Braek, S. Paoletti, I. Donati, On the initial binding of alginate by calcium ions. The tilted egg-box hypothesis, J Phys Chem B 117(24) (2013) 7277-82.

[222] J. Jang, Y.J. Seol, H.J. Kim, J. Kundu, S.W. Kim, D.W. Cho, Effects of alginate hydrogel cross-linking density on mechanical and biological behaviors for tissue engineering, J Mech Behav Biomed Mater 37C (2014) 69-77.

[223] S.K. Tam, J. Dusseault, S. Bilodeau, G. Langlois, J.P. Halle, L. Yahia, Factors influencing alginate gel biocompatibility, J Biomed Mater Res A 98(1) (2011) 40-52.

[224] T.A. Becker, D.R. Kipke, T. Brandon, Calcium alginate gel: a biocompatible and mechanically stable polymer for endovascular embolization, Journal of Biomedical Materials Research 54(1) (2001) 76-86.

[225] J.M. Szymanski, A.W. Feinberg, Fabrication of freestanding alginate microfibers and microstructures for tissue engineering applications, Biofabrication 6(2) (2014) 024104.

[226] Y. Luo, A. Lode, M. Gelinsky, Direct plotting of three-dimensional hollow fiber scaffolds based on concentrated alginate pastes for tissue engineering, Adv Healthc Mater 2(6) (2013) 777-83.

[227] H. Tan, J.P. Rubin, K.G. Marra, Injectable in situ forming biodegradable chitosan-hyaluronic acid based hydrogels for adipose tissue regeneration, Organogenesis 6(3) (2010) 173-80.

[228] K.Y. Lee, H.J. Kong, R.G. Larson, D.J. Mooney, Hydrogel formation via cell crosslinking, Advanced Materials 15(21) (2003) 1828-1832.

[229] H. Tan, J.P. Rubin, K.G. Marra, Direct synthesis of biodegradable polysaccharide derivative hydrogels through aqueous Diels-Alder chemistry, Macromol Rapid Commun 32(12) (2011) 905-11.

[230] R.H. Schmedlen, K.S. Masters, J.L. West, Photocrosslinkable polyvinyl alcohol hydrogels that can be modified with cell adhesion peptides for use in tissue engineering, Biomaterials 23(22) (2002) 4325-32.

[231] J.C. Sun, H.P. Tan, Alginate-Based Biomaterials for Regenerative Medicine Applications, Materials 6(4) (2013) 1285-1309.

[232] X. Huang, X. Zhang, X. Wang, C. Wang, B. Tang, Microenvironment of alginate-based microcapsules for cell culture and tissue engineering, Journal of Bioscience and Bioengineering 114(1) (2012) 1-8.

[233] U. Hersel, C. Dahmen, H. Kessler, RGD modified polymers: biomaterials for stimulated cell adhesion and beyond, Biomaterials 24(24) (2003) 4385-415.

[234] S.I. Jeong, M.D. Krebs, C.A. Bonino, S.A. Khan, E. Alsberg, Electrospun alginate nanofibers with controlled cell adhesion for tissue engineering, Macromolecular Bioscience 10(8) (2010) 934-43.

[235] J.A. Rowley, G. Madlambayan, D.J. Mooney, Alginate hydrogels as synthetic extracellular matrix materials, Biomaterials 20(1) (1999) 45-53.

[236] S. Mazzitelli, L. Capretto, D. Carugo, X. Zhang, R. Piva, C. Nastruzzi, Optimised production of multifunctional microfibres by microfluidic chip technology for tissue engineering applications, Lab Chip (2011).

[237] C.S. Tan, A. Jejurikar, B. Rai, T. Bostrom, G. Lawrie, L. Grondahl, Encapsulation of a glycosaminoglycan in hydroxyapatite/alginate capsules, J Biomed Mater Res A 91(3) (2009) 866-77.

[238] C. Branco da Cunha, D.D. Klumpers, W.A. Li, S.T. Koshy, J.C. Weaver, O. Chaudhuri, P.L. Granja, D.J. Mooney, Influence of the stiffness of three-dimensional alginate/collagen-I interpenetrating networks on fibroblast biology, Biomaterials (2014).

[239] P. Zhao, C. Deng, H. Xu, X. Tang, H. He, C. Lin, J. Su, Fabrication of photo-crosslinked chitosan- gelatin scaffold in sodium alginate hydrogel for chondrocyte culture, Biomed Mater Eng 24(1) (2014) 633-41.

[240] J. Venkatesan, I. Bhatnagar, P. Manivasagan, K.H. Kang, S.K. Kim, Alginate composites for bone tissue engineering: A review, International Journal of Biological Macromolecules (2014).

[241] J.K. Gandhi, E.C. Opara, E.M. Brey, Alginate-based strategies for therapeutic vascularization, Ther Deliv 4(3) (2013) 327-41.

[242] A. Garate, A. Murua, G. Orive, R.M. Hernandez, J.L. Pedraz, Stem cells in alginate bioscaffolds, Ther Deliv 3(6) (2012) 761-74.

[243] K.D. Rane, D.G. Hoover, Production of Chitosan by Fungi, Food Biotechnol 7(1) (1993) 11-33.

[244] M. Rinaudo, Chitin and chitosan: Properties and applications, Prog Polym Sci 31(7) (2006) 603-632.

[245] F. Croisier, C. Jerome, Chitosan-based biomaterials for tissue engineering, Eur Polym J 49(4) (2013) 780-792.

[246] R. Jayakumar, D. Menon, K. Manzoor, S.V. Nair, H. Tamura, Biomedical applications of chitin and chitosan based nanomaterials-A short review, Carbohydrate Polymers 82(2) (2010) 227-232.

[247] N. Acosta, C. Jimenez, V. Borau, A. Heras, Extraction and Characterization of Chitin from Crustaceans, Biomass Bioenerg 5(2) (1993) 145-153.

[248] M.R. Leedy, H.J. Martin, P.A. Norowski, J.A. Jennings, W.O. Haggard, J.D. Bumgardner, Use of Chitosan as a Bioactive Implant Coating for Bone-Implant Applications, Chitosan for Biomaterials Ii 244 (2011) 129-165.

[249] F.J. Pavinatto, L. Caseli, O.N. Oliveira, Chitosan in Nanostructured Thin Films, Biomacromolecules 11(8) (2010) 1897-1908.

[250] H. Wu, Y. Wan, S. Dalai, R. Zhang, Response of rat osteoblasts to polycaprolactone/chitosan blend porous scaffolds, J Biomed Mater Res A 92(1) (2010) 238-45.

[251] S. Bagheri-Khoulenjani, S.M. Taghizadeh, H. Mirzadeh, An investigation on the short-term biodegradability of chitosan with various molecular weights and degrees of deacetylation, Carbohydrate Polymers 78(4) (2009) 773-778.

[252] M. Prabaharan, Review paper: chitosan derivatives as promising materials for controlled drug delivery, J Biomater Appl 23(1) (2008) 5-36.

[253] R. Raftery, F.J. O'Brien, S.A. Cryan, Chitosan for gene delivery and orthopedic tissue engineering applications, Molecules 18(5) (2013) 5611-47.

[254] I.Y. Kim, S.J. Seo, H.S. Moon, M.K. Yoo, I.Y. Park, B.C. Kim, C.S. Cho, Chitosan and its derivatives for tissue engineering applications, Biotechnol Adv 26(1) (2008) 1-21.

[255] A. Francesko, T. Tzanov, Chitin, chitosan and derivatives for wound healing and tissue engineering, Adv Biochem Eng Biotechnol 125 (2011) 1-27.

[256] M. Rajam, S. Pulavendran, C. Rose, A.B. Mandal, Chitosan nanoparticles as a dual growth factor delivery system for tissue engineering applications, International Journal of Pharmaceutics (2011).

[257] C. Correia, L.S. Moreirateixeira, L. Moroni, R.L. Reis, C. van Blitterswijk, M. Karperien, J. Mano, Chitosan Scaffolds Containing Hyaluronic Acid for Cartilage Tissue Engineering, Tissue Eng Part C Methods (2011).

[258] G. Sun, J.J. Mao, Engineering dextran-based scaffolds for drug delivery and tissue repair, Nanomedicine 7(11) (2012) 1771-84.

[259] R. Jin, L.S. Moreira Teixeira, P.J. Dijkstra, Z. Zhong, C.A. van Blitterswijk, M. Karperien, J. Feijen, Enzymatically crosslinked dextran-tyramine hydrogels as injectable scaffolds for cartilage tissue engineering, Tissue Eng Part A 16(8) (2010) 2429-40.

[260] Y. Liu, M.B. Chan-Park, Hydrogel based on interpenetrating polymer networks of dextran and gelatin for vascular tissue engineering, Biomaterials 30(2) (2009) 196-207.

[261] A. Martins, S. Chung, A.J. Pedro, R.A. Sousa, A.P. Marques, R.L. Reis, N.M. Neves, Hierarchical starch-based fibrous scaffold for bone tissue engineering applications, Journal of Tissue Engineering and Regenerative Medicine 3(1) (2009) 37-42.

[262] N. Reddy, Y. Yang, Preparation and properties of starch acetate fibers for potential tissue engineering applications, Biotechnol Bioeng 103(5) (2009) 1016-22.

[263] D. Pasqui, P. Torricelli, M. De Cagna, M. Fini, R. Barbucci, Carboxymethyl cellulose-hydroxyapatite hybrid hydrogel as a composite material for bone tissue engineering applications, J Biomed Mater Res A 102(5) (2014) 1568-79.

[264] N. Ninan, M. Muthiah, I.K. Park, A. Elain, S. Thomas, Y. Grohens, Pectin/carboxymethyl cellulose/microfibrillated cellulose composite scaffolds for tissue engineering, Carbohydrate Polymers 98(1) (2013) 877-85.

[265] F. Munarin, S.G. Guerreiro, M.A. Grellier, M.C. Tanzi, M.A. Barbosa, P. Petrini, P.L. Granja, Pectin-based injectable biomaterials for bone tissue engineering, Biomacromolecules 12(3) (2011) 568-77.

[266] S. Van Vlierberghe, P. Dubruel, E. Schacht, Biopolymer-Based Hydrogels As Scaffolds for Tissue Engineering Applications: A Review, Biomacromolecules (2011).

[267] E. Chevalier, D. Chulia, C. Pouget, M. Viana, Fabrication of porous substrates: a review of processes using pore forming agents in the biomaterial field, J Pharm Sci 97(3) (2008) 1135-54.

[268] J. Guan, J.J. Stankus, W.R. Wagner, Development of Composite Porous Scaffolds Based on Collagen and Biodegradable Poly(ester Urethane)urea, Cell Transplantation 15 (2006) S17-S27.

[269] W. Sun, D.M. Tiemessen, M. Sloff, R.J. Lammers, E.L. de Mulder, J. Hilborn, B. Gupta, W.F. Feitz, W.F. Daamen, T.H. van Kuppevelt, P.J. Geutjes, E. Oosterwijk, Improving the Cell Distribution in Collagen-Coated Poly-Caprolactone Knittings, Tissue Eng Part C Methods (2012).

[270] J.M. Snowden, D.A. Swann, Vitreous structure. V. The morphology and thermal stability of vitreous collagen fibers and comparison to articular cartilage (type II) collagen, Invest Ophthalmol Vis Sci 19(6) (1980) 610-8.

[271] T. Johnson, R. Bahrampourian, A. Patel, K. Mequanint, Fabrication of highly porous tissue-engineering scaffolds using selective spherical porogens, Biomed Mater Eng 20(2) (2010) 107-18.

[272] X. Shen, L. Chen, X. Cai, T. Tong, H. Tong, J. Hu, A novel method for the fabrication of homogeneous hydroxyapatite/collagen nanocomposite and nanocomposite scaffold with hierarchical porosity, J Mater Sci Mater Med 22(2) (2011) 299-305.

[273] H.W. Jun, J.L. West, Endothelialization of microporous YIGSR/PEG-modified polyurethaneurea, Tissue Engineering 11(7-8) (2005) 1133-40.

[274] J.I. Lim, Y.K. Lee, J.S. Shin, K.J. Lim, Preparation of Interconnected Porous Chitosan Scaffolds by Sodium Acetate Particulate Leaching, J Biomater Sci Polym Ed (2010).

[275] L. Buttafoco, P. Engbers-Buijtenhuijs, A.A. Poot, P.J. Dijkstra, W.F. Daamen, T.H. van Kuppevelt, I. Vermes, J. Feijen, First steps towards tissue engineering of small-diameter blood vessels: preparation of flat scaffolds of collagen and elastin by means of freeze drying, J Biomed Mater Res B Appl Biomater 77(2) (2006) 357-68.

[276] F.P. Melchels, J. Feijen, D.W. Grijpma, A review on stereolithography and its applications in biomedical engineering, Biomaterials 31(24) (2010) 6121-30.

[277] T.M. Hsieh, C.W. Ng, K. Narayanan, A.C. Wan, J.Y. Ying, Three-dimensional microstructured tissue scaffolds fabricated by two-photon laser scanning photolithography, Biomaterials 31(30) (2010) 7648-52.

[278] K.A. Faraj, T.H. van Kuppevelt, W.F. Daamen, Construction of collagen scaffolds that mimic the three-dimensional architecture of specific tissues, Tissue Engineering 13(10) (2007) 2387-94.

[279] F.J. O'Brien, B.A. Harley, I.V. Yannas, L. Gibson, Influence of freezing rate on pore structure in freeze-dried collagen-GAG scaffolds, Biomaterials 25(6) (2004) 1077-86.

[280] K.M. Brouwer, W.F. Daamen, H.R. Hoogenkamp, P.J. Geutjes, I. de Blaauw, W. Janssen-Kessels, W. de Boode, E. Versteeg, R.M. Wijnen, W.F. Feitz, M. Wijnen, T.H. van Kuppevelt, Collagen-Vicryl scaffolds for reconstruction of the diaphragm in a large animal model, J Biomed Mater Res B Appl Biomater 102(4) (2014) 756-63.

[281] K. Park, W.C.W. Shalaby, H. Park, Hydrogels, Definition, Hydrogel as a Biomaterial, Biodegradable Hydrogels, Biodegradation, Technomic, Lancaster, 1993.

[282] D.L. Elbert, Liquid-liquid two-phase systems for the production of porous hydrogels and hydrogel microspheres for biomedical applications: A tutorial review, Acta Biomaterialia 7(1) (2011) 31-56.

[283] A.M. Kloxin, C.J. Kloxin, C.N. Bowman, K.S. Anseth, Mechanical properties of cellularly responsive hydrogels and their experimental determination, Advanced Materials 22(31) (2010) 3484-94.

[284] E.S. Lai, C.M. Anderson, G.G. Fuller, Designing a tubular matrix of oriented collagen fibrils for tissue engineering, Acta Biomaterialia (2011).

[285] L. Fassina, E. Saino, L. Visai, M.A. Avanzini, M.G. Cusella De Angelis, F. Benazzo, S. Van Vlierberghe, P. Dubruel, G. Magenes, Use of a gelatin cryogel as biomaterial scaffold in the differentiation process of human bone marrow stromal cells, Conf Proc IEEE Eng Med Biol Soc 2010 (2010) 247-50.

[286] S.E. Dunphy, J.A. Bratt, K.M. Akram, N.R. Forsyth, A.J. El Haj, Hydrogels for lung tissue engineering: Biomechanical properties of thin collagen-elastin constructs, J Mech Behav Biomed Mater (2014).

[287] S.J. Bidarra, C.C. Barrias, P.L. Granja, Injectable alginate hydrogels for cell delivery in tissue engineering, Acta Biomaterialia 10(4) (2014) 1646-62.

[288] M.A. de Moraes, E. Paternotte, D. Mantovani, M.M. Beppu, Mechanical and biological performances of new scaffolds made of collagen hydrogels and fibroin microfibers for vascular tissue engineering, Macromolecular Bioscience 12(9) (2012) 1253-64.

[289] Z. Yue, F. Wen, S. Gao, M.Y. Ang, P.K. Pallathadka, L. Liu, H. Yu, Preparation of three-dimensional interconnected macroporous cellulosic hydrogels for soft tissue engineering, Biomaterials 31(32) (2010) 8141-52.

[290] C. Arakawa, R. Ng, S. Tan, S. Kim, B. Wu, M. Lee, Photopolymerizable chitosan-collagen hydrogels for bone tissue engineering, Journal of Tissue Engineering and Regenerative Medicine (2014).

[291] G.Y. Huang, L.H. Zhou, Q.C. Zhang, Y.M. Chen, W. Sun, F. Xu, T.J. Lu, Microfluidic hydrogels for tissue engineering, Biofabrication 3(1) (2011) 012001.

[292] A. Sharma, S. Bhat, T. Vishnoi, V. Nayak, A. Kumar, Three-dimensional supermacroporous carrageenan-gelatin cryogel matrix for tissue engineering applications, Biomed Res Int 2013 (2013) 478279.

[293] S.A. Maskarinec, D.A. Tirrell, Protein engineering approaches to biomaterials design, Curr Opin Biotechnol 16(4) (2005) 422-6.

[294] S. Van Vlierberghe, P. Dubruel, E. Schacht, Biopolymer-based hydrogels as scaffolds for tissue engineering applications: a review, Biomacromolecules 12(5) (2011) 1387-408.

[295] P.S. Robinson, S.L. Johnson, M.C. Evans, V.H. Barocas, R.T. Tranquillo, Functional tissue-engineered valves from cell-remodeled fibrin with commissural alignment of cell-produced collagen, Tissue Eng Part A 14(1) (2008) 83-95.

[296] J.W. Bae, J.H. Choi, Y. Lee, K.D. Park, Horseradish peroxidase-catalysed in situ-forming hydrogels for tissue-engineering applications, Journal of Tissue Engineering and Regenerative Medicine (2014).

[297] A.L. Daniel-da-Silva, R.S. Carvalho, T. Trindade, Magnetic hydrogel nanocomposites and composite nanoparticles--a review of recent patented works, Recent Pat Nanotechnol 7(2) (2013) 153-66.

[298] T. Billiet, M. Vandenhaute, J. Schelfhout, S. Van Vlierberghe, P. Dubruel, A review of trends and limitations in hydrogel-rapid prototyping for tissue engineering, Biomaterials 33(26) (2012) 6020-41.

[299] R.A. Siegel, E.E. Nuxoll, M.A. Hillmyer, B. Ziaie, Top-down and bottom-up fabrication techniques for hydrogel based sensing and hormone delivery microdevices, Conf Proc IEEE Eng Med Biol Soc 2009 (2009) 232-5.

[300] J. Liu, H. Song, L. Zhang, H. Xu, X. Zhao, Self-assembly-peptide hydrogels as tissue-engineering scaffolds for three-dimensional culture of chondrocytes in vitro, Macromolecular Bioscience 10(10) (2010) 1164-70.

[301] F.J. Pavinatto, L. Caseli, O.N. Oliveira, Chitosan in nanostructured thin films, Biomacromolecules 11(8) (2010) 1897-908.

[302] C.L. Bowe, L. Mokhtarzadeh, P. Venkatesan, S. Babu, H.R. Axelrod, M.J. Sofia, R. Kakarla, T.Y. Chan, J.S. Kim, H.J. Lee, G.L. Amidon, S.Y. Choe, S. Walker, D. Kahne, Design of compounds that increase the absorption of polar molecules, Proc Natl Acad Sci U S A 94(22) (1997) 12218-12223.

[303] T. Boudou, T. Crouzier, K. Ren, G. Blin, C. Picart, Multiple functionalities of polyelectrolyte multilayer films: new biomedical applications, Advanced Materials 22(4) (2010) 441-67.

[304] D.T. Haynie, L. Zhang, W. Zhao, J.S. Rudra, Protein-inspired multilayer nanofilms: science, technology and medicine, Nanomedicine 2(3) (2006) 150-7.

[305] S.A. Shi, X.H. Wang, G. Guo, M. Fan, M.J. Huang, Z.Y. Qian, Preparation and characterization of microporous poly(D,L-lactic acid) film for tissue engineering scaffold, Int J Nanomedicine 5 (2010) 1049-1055.

[306] R. Truckenmuller, S. Giselbrecht, N. Rivron, E. Gottwald, V. Saile, A. van den Berg, M. Wessling, C. van Blitterswijk, Thermoforming of film-based biomedical microdevices, Advanced Materials 23(11) (2011) 1311-29.

[307] F. Taraballi, S. Zanini, C. Lupo, S. Panseri, C. Cunha, C. Riccardi, M. Marcacci, M. Campione, L. Cipolla, Amino and carboxyl plasma functionalization of collagen films for tissue engineering applications, J Colloid Interface Sci 394 (2013) 590-7.

[308] Y. Liu, L. Ren, Y.J. Wang, Crosslinked collagen-gelatin-hyaluronic acid biomimetic film for cornea tissue engineering applications, Mat Sci Eng C-Mater 33(1) (2013) 196-201.

[309] E. Rosellini, C. Cristallini, N. Barbani, G. Vozzi, P. Giusti, Preparation and characterization of alginate/gelatin blend films for cardiac tissue engineering, J Biomed Mater Res A 91(2) (2009) 447-53.

[310] Y. Liu, L. Ren, K. Long, L. Wang, Y.J. Wang, Preparation and characterization of a novel tobramycin-containing antibacterial collagen film for corneal tissue engineering, Acta Biomaterialia 10(1) (2014) 289-299.

[311] S. Wrobel, S.C. Serra, S. Ribeiro-Samy, N. Sousa, C. Heimann, C. Barwig, C. Grothe, A.J. Salgado, K. Haastert-Talini, In Vitro Evaluation of Cell-Seeded Chitosan Films for Peripheral Nerve Tissue Engineering, Tissue Eng Part A (2014).

[312] M.K. Yeh, Y.M. Liang, K.M. Cheng, N.T. Dai, C.C. Liu, J.J. Young, A novel cell support membrane for skin tissue engineering: Gelatin film cross-linked with 2-chloro-1-methylpyridinium iodide, Polymer 52(4) (2011) 996-1003.

[313] T. Chlapanidas, M.C. Tosca, S. Farago, S. Perteghella, M. Galuzzi, G. Lucconi, B. Antonioli, F. Ciancio, V. Rapisarda, D. Vigo, M. Marazzi, M. Faustini, M.L. Torre, Formulation and characterization of silk fibroin films as a scaffold for adipose-derived stem cells in skin tissue engineering, Int J Immunopathol Pharmacol 26(1 Suppl) (2013) 43-9.

[314] G. Lammers, G. Roth, M. Heck, R. Zengerle, G.S. Tjabringa, E.M. Versteeg, T. Hafmans, R. Wismans, D.P. Reinhardt, E.T. Verwiel, P.L. Zeeuwen, J. Schalkwijk, R. Brock, W.F. Daamen, T.H. van Kuppevelt, Construction of a Microstructured Collagen Membrane Mimicking the Papillary Dermis Architecture and Guiding Keratinocyte Morphology and Gene Expression, Macromolecular Bioscience (2012).

[315] L.W. Tien, E.S. Gil, S.H. Park, B.B. Mandal, D.L. Kaplan, Patterned Silk Film Scaffolds for Aligned Lamellar Bone Tissue Engineering, Macromolecular Bioscience 12(12) (2012) 1671-1679.

[316] W. Chen, Y. Tabata, Y.W. Tong, Fabricating tissue engineering scaffolds for simultaneous cell growth and drug delivery, Curr Pharm Des 16(21) (2010) 2388-94.

[317] S.H. Wibowo, A. Sulistio, E.H. Wong, A. Blencowe, G.G. Qiao, Polypeptide films via N-carboxyanhydride ring-opening polymerization (NCA-ROP): past, present and future, Chem Commun (Camb) 50(39) (2014) 4971-88.

[318] A.C. Vieira, J.C. Vieira, J.M. Ferra, F.D. Magalhaes, R.M. Guedes, A.T. Marques, Mechanical study of PLA-PCL fibers during in vitro degradation, J Mech Behav Biomed Mater 4(3) (2011) 451-60.

[319] B. Klosterhalfen, K. Junge, U. Klinge, The lightweight and large porous mesh concept for hernia repair, Expert Reviews in Medical Devices 2(1) (2005) 1-15.

[320] D.I. Zeugolis, S.T. Khew, E.S. Yew, A.K. Ekaputra, Y.W. Tong, L.Y. Yung, D.W. Hutmacher, C. Sheppard, M. Raghunath, Electro-spinning of pure collagen nano-fibres - just an expensive way to make gelatin?, Biomaterials 29(15) (2008) 2293-305.

[321] R. Murugan, S. Ramakrishna, Nano-featured scaffolds for tissue engineering: a review of spinning methodologies, Tissue Engineering 12(3) (2006) 435-47.

[322] V. Milleret, B. Simona, P. Neuenschwander, H. Hall, Tuning electrospinning parameters for production of 3D-fiber-fleeces with increased porosity for soft tissue engineering applications, Eur Cell Mater 21 (2011) 286-303.

[323] H. Chen, X. Fan, J. Xia, P. Chen, X. Zhou, J. Huang, J. Yu, P. Gu, Electrospun chitosan-graft-poly (varepsilon-caprolactone)/poly (varepsilon-caprolactone) nanofibrous scaffolds for retinal tissue engineering, Int J Nanomedicine 6 (2011) 453-61.

[324] H. Liu, X. Li, G. Zhou, H. Fan, Y. Fan, Electrospun sulfated silk fibroin nanofibrous scaffolds for vascular tissue engineering, Biomaterials 32(15) (2011) 3784-93.

[325] S. Kanokpanont, S. Damrongsakkul, J. Ratanavaraporn, P. Aramwit, Physico-chemical properties and efficacy of silk fibroin fabric coated with different waxes as wound dressing, International Journal of Biological Macromolecules 55 (2013) 88-97.

[326] D. Enea, F. Henson, S. Kew, J. Wardale, A. Getgood, R. Brooks, N. Rushton, Extruded collagen fibres for tissue engineering applications: effect of crosslinking method on mechanical and biological properties, J Mater Sci Mater Med 22(6) (2011) 1569-78.

[327] I. Kanatani, A. Kanematsu, Y. Inatsugu, M. Imamura, H. Negoro, N. Ito, S. Yamamoto, Y. Tabata, Y. Ikada, O. Ogawa, Fabrication of an optimal urethral graft using collagen-sponge tubes reinforced with Copoly(L-lactide/epsilon-caprolactone) fabric, Tissue Engineering 13(12) (2007) 2933-40.

[328] J. Huo, R. Rojas, J. Bohlin, J. Hilborn, E.K. Gamstedt, Parametric elastic analysis of coupled helical coils for tubular implant applications: experimental characterization and numerical analysis, J Mech Behav Biomed Mater 29 (2014) 462-9.

[329] H.R. Hoogenkamp, M.J. Koens, P.J. Geutjes, H. Ainoedhofer, G. Wanten, D.M. Tiemessen, J. Hilborn, B. Gupta, W.F. Feitz, W.F. Daamen, A.K. Saxena, E. Oosterwijk, T.H. van Kuppevelt, Seamless vascularized large-diameter tubular collagen scaffolds reinforced with polymer knittings for esophageal regenerative medicine, Tissue Eng Part C Methods 20(5) (2014) 423-30.

[330] D.W. Hutmacher, S. Cool, Concepts of scaffold-based tissue engineering-the rationale to use solid free-form fabrication techniques, J Cell Mol Med 11(4) (2007) 654-669.

[331] S.M. Peltola, F.P.W. Melchels, D.W. Grijpma, M. Kellomaki, A review of rapid prototyping techniques for tissue engineering purposes, Ann Med 40(4) (2008) 268-280.

[332] F. Rengier, A. Mehndiratta, H. von Tengg-Kobligk, C.M. Zechmann, R. Unterhinninghofen, H.U. Kauczor, F.L. Giesel, 3D printing based on imaging data: review of medical applications, Int J Comput Assist Radiol Surg 5(4) (2010) 335-41.

[333] D.J. Richards, Y. Tan, J. Jia, H. Yao, Y. Mei, 3D Printing for Tissue Engineering, Isr J Chem 53(9-10) (2013) 805-814.

[334] J.A. Paten, G.E. Tilburey, E.A. Molloy, R. Zareian, C.V. Trainor, J.W. Ruberti, Utility of an optically-based, micromechanical system for printing collagen fibers, Biomaterials 34(11) (2013) 2577-87.

[335] S.A. Skoog, P.L. Goering, R.J. Narayan, Stereolithography in tissue engineering, J Mater Sci-Mater M 25(3) (2014) 845-856.

[336] C.H. Chen, V.B. Shyu, J.P. Chen, M.Y. Lee, Selective laser sintered poly-epsilon-caprolactone scaffold hybridized with collagen hydrogel for cartilage tissue engineering, Biofabrication 6(1) (2014) 015004.

[337] Y. Xuan, H. Tang, B. Wu, X. Ding, Z. Lu, W. Li, Z. Xu, A specific groove design for individualized healing in a canine partial sternal defect model by a polycaprolactone/hydroxyapatite scaffold coated with bone marrow stromal cells, J Biomed Mater Res A 102(10) (2014) 3401-8.

[338] J. Giannatsis, V. Dedoussis, Additive fabrication technologies applied to medicine and health care: a review, Int J Adv Manuf Tech 40(1-2) (2009) 116-127.

[339] A.M. Kasko, D.Y. Wong, Two-photon lithography in the future of cell-based therapeutics and regenerative medicine: a review of techniques for hydrogel patterning and controlled release, Future Med Chem 2(11) (2010) 1669-80.

[340] Y.B. Lee, S. Polio, W. Lee, G. Dai, L. Menon, R.S. Carroll, S.S. Yoo, Bio-printing of collagen and VEGF-releasing fibrin gel scaffolds for neural stem cell culture, Exp Neurol 223(2) (2010) 645-52.

[341] T. Billiet, E. Gevaert, T. De Schryver, M. Cornelissen, P. Dubruel, The 3D printing of gelatin methacrylamide cell-laden tissue-engineered constructs with high cell viability, Biomaterials 35(1) (2014) 49-62.

[342] S.H. Hashimdeen, M. Miodownik, M.J. Edirisinghe, Print head design and control for electrohydrodynamic printing of silk fibroin, Mater Sci Eng C Mater Biol Appl 33(6) (2013) 3309-18.

[343] M.T. Poldervaart, H. Wang, J. van der Stok, H. Weinans, S.C. Leeuwenburgh, F.C. Oner, W.J. Dhert, J. Alblas, Sustained release of BMP-2 in bioprinted alginate for osteogenicity in mice and rats, PLoS One 8(8) (2013) e72610.

[344] J.A. Phillippi, E. Miller, L. Weiss, J. Huard, A. Waggoner, P. Campbell, Microenvironments engineered by inkjet bioprinting spatially direct adult stem cells toward muscle- and bone-like subpopulations, Stem Cells 26(1) (2008) 127-34.

[345] B.R. Ringeisen, C.M. Othon, J.A. Barron, D. Young, B.J. Spargo, Jet-based methods to print living cells, Biotechnol J 1(9) (2006) 930-48.

[346] C.Z. Liu, Z.D. Xia, Z.W. Han, P.A. Hulley, J.T. Triffitt, J.T. Czernuszka, Novel 3D collagen scaffolds fabricated by indirect printing technique for tissue engineering, J Biomed Mater Res B Appl Biomater 85(2) (2008) 519-28.

[347] V. Mironov, V. Kasyanov, C. Drake, R.R. Markwald, Organ printing: promises and challenges, Regen Med 3(1) (2008) 93-103.

[348] T. Boland, V. Mironov, A. Gutowska, E.A. Roth, R.R. Markwald, Cell and organ printing 2: fusion of cell aggregates in three-dimensional gels, Anat Rec A Discov Mol Cell Evol Biol 272(2) (2003) 497-502.

[349] L. Koch, A. Deiwick, S. Schlie, S. Michael, M. Gruene, V. Coger, D. Zychlinski, A. Schambach, K. Reimers, P.M. Vogt, B. Chichkov, Skin tissue generation by laser cell printing, Biotechnol Bioeng 109(7) (2012) 1855-63.

[350] I.T. Ozbolat, Y. Yu, Bioprinting toward organ fabrication: challenges and future trends, IEEE Trans Biomed Eng 60(3) (2013) 691-9.

[351] F. Marga, K. Jakab, C. Khatiwala, B. Shepherd, S. Dorfman, B. Hubbard, S. Colbert, F. Gabor, Toward engineering functional organ modules by additive manufacturing, Biofabrication 4(2) (2012) 022001.

[352] T. Gilbert, T. Sellaro, S. Badylak, Decellularization of tissues and organs, Biomaterials (2006).

[353] P.M. Crapo, T.W. Gilbert, S.F. Badylak, An overview of tissue and whole organ decellularization processes, Biomaterials 32(12) (2011) 3233-43.

[354] A.F. Pellegata, M.A. Asnaghi, I. Stefani, A. Maestroni, S. Maestroni, T. Dominioni, S. Zonta, G. Zerbini, S. Mantero, Detergent-enzymatic decellularization of Swine blood vessels: insight on mechanical properties for vascular tissue engineering, Biomed Res Int 2013 (2013) 918753.

[355] A. Bader, T. Schilling, O.E. Teebken, G. Brandes, T. Herden, G. Steinhoff, A. Haverich, Tissue engineering of heart valves--human endothelial cell seeding of detergent acellularized porcine valves, Eur J Cardiothorac Surg 14(3) (1998) 279-84.

[356] S. Cayan, C. Chermansky, N. Schlote, N. Sekido, L. Nunes, R. Dahiya, E.A. Tanagho, The bladder acellular matrix graft in a rat chemical cystitis model: functional and histologic evaluation, The Journal of Urology 168(2) (2002) 798-804.

[357] C. Booth, S.A. Korossis, H.E. Wilcox, K.G. Watterson, J.N. Kearney, J. Fisher, E. Ingham, Tissue engineering of cardiac valve prostheses I: development and histological characterization of an acellular porcine scaffold, J Heart Valve Dis 11(4) (2002) 457-62.

[358] T.W. Gilbert, S. Wognum, E.M. Joyce, D.O. Freytes, M.S. Sacks, S.F. Badylak, Collagen fiber alignment and biaxial mechanical behavior of porcine urinary bladder derived extracellular matrix, Biomaterials 29(36) (2008) 4775-82.

[359] I. Prasertsung, S. Kanokpanont, T. Bunaprasert, V. Thanakit, S. Damrongsakkul, Development of acellular dermis from porcine skin using periodic pressurized technique, J Biomed Mater Res B Appl Biomater 85(1) (2008) 210-9.

[360] C.C. Xu, R.W. Chan, N. Tirunagari, A biodegradable, acellular xenogeneic scaffold for regeneration of the vocal fold lamina propria, Tissue Engineering 13(3) (2007) 551-66.

[361] R.J. Levy, N. Vyavahare, M. Ogle, P. Ashworth, R. Bianco, F.J. Schoen, Inhibition of cusp and aortic wall calcification in ethanol- and aluminum-treated bioprosthetic heart valves in sheep: background, mechanisms, and synergism, J Heart Valve Dis 12(2) (2003) 209-16; discussion 216.

[362] J. Gailit, E. Ruoslahti, Regulation of the fibronectin receptor affinity by divalent cations, J Biol Chem 263(26) (1988) 12927-32.

[363] B.T. Yu, W.T. Li, B.Q. Song, Y.L. Wu, Comparative study of the Triton X-100-sodium deoxycholate method and detergent-enzymatic digestion method for decellularization of porcine aortic valves, Eur Rev Med Pharmacol Sci 17(16) (2013) 2179-84.

[364] A.R. Gillies, L.R. Smith, R.L. Lieber, S. Varghese, Method for decellularizing skeletal muscle without detergents or proteolytic enzymes, Tissue Eng Part C Methods 17(4) (2011) 383-9.

[365] J.C. Fitzpatrick, P.M. Clark, F.M. Capaldi, Effect of decellularization protocol on the mechanical behavior of porcine descending aorta, Int J Biomater 2010 (2010).

[366] S.H. Hung, C.H. Su, F.P. Lee, H. Tseng, Larynx decellularization: combining freeze-drying and sonication as an effective method, J Voice 27(3) (2013) 289-94.

[367] J. Negishi, S. Funamoto, T. Kimura, K. Nam, T. Higami, A. Kishida, Porcine radial artery decellularization by high hydrostatic pressure, Journal of Tissue Engineering and Regenerative Medicine (2012).

[368] K. Sawada, D. Terada, T. Yamaoka, S. Kitamura, T. Fujisato, Cell removal with supercritical carbon dioxide for acellular artificial tissue, Journal of Chemical Technology and Biotechnology 83(6) (2008) 943-949.

[369] J. Burk, I. Erbe, D. Berner, J. Kacza, C. Kasper, B. Pfeiffer, K. Winter, W. Brehm, Freeze-Thaw Cycles Enhance Decellularization of Large Tendons, Tissue Eng Part C Methods (2013).

[370] P.W. Henderson, V.V. Nagineni, A. Harper, N. Bavinck, A.M. Sohn, D.D. Krijgh, N. Jimenez, A.L. Weinstein, J.A. Spector, Development of an acellular bioengineered matrix with a dominant vascular pedicle, J Surg Res 164(1) (2010) 1-5.

[371] A. Hopkinson, V.A. Shanmuganathan, T. Gray, A.M. Yeung, J. Lowe, D.K. James, H.S. Dua, Optimization of amniotic membrane (AM) denuding for tissue engineering, Tissue Eng Part C Methods 14(4) (2008) 371-81.

[372] B. Mendoza-Novelo, E.E. Avila, J.V. Cauich-Rodriguez, E. Jorge-Herrero, F.J. Rojo, G.V. Guinea, J.L. Mata-Mata, Decellularization of pericardial tissue and its impact on tensile viscoelasticity and glycosaminoglycan content, Acta Biomaterialia 7(3) (2011) 1241-8.

[373] N. Hou, P. Cui, J. Luo, R. Ma, L. Zhu, Tissue-engineered larynx using perfusion-decellularized technique and mesenchymal stem cells in a rabbit model, Acta Otolaryngol (2011).

[374] Y. Zhao, S. Zhang, J. Zhou, J. Wang, M. Zhen, Y. Liu, J. Chen, Z. Qi, The development of a tissue-engineered artery using decellularized scaffold and autologous ovine mesenchymal stem cells, Biomaterials 31(2) (2010) 296-307.

[375] K.H. Nakayama, C.A. Batchelder, C.I. Lee, A.F. Tarantal, Decellularized rhesus monkey kidney as a three-dimensional scaffold for renal tissue engineering, Tissue Eng Part A 16(7) (2010) 2207-16.

[376] J.H. Ingram, S. Korossis, G. Howling, J. Fisher, E. Ingham, The use of ultrasonication to aid recellularization of acellular natural tissue scaffolds for use in anterior cruciate ligament reconstruction, Tissue Engineering 13(7) (2007) 1561-72.

[377] H.C. Ott, T.S. Matthiesen, S.K. Goh, L.D. Black, S.M. Kren, T.I. Netoff, D.A. Taylor, Perfusion-decellularized matrix: using nature's platform to engineer a bioartificial heart, Nat Med 14(2) (2008) 213-21.

[378] J.M. Singelyn, J.A. DeQuach, S.B. Seif-Naraghi, R.B. Littlefield, P.J. Schup-Magoffin, K.L. Christman, Naturally derived myocardial matrix as an injectable scaffold for cardiac tissue engineering, Biomaterials 30(29) (2009) 5409-16.

[379] J.S. Choi, J.K. Williams, M. Greven, K.A. Walter, P.W. Laber, G. Khang, S. Soker, Bioengineering endothelialized neo-corneas using donor-derived corneal endothelial cells and decellularized corneal stroma, Biomaterials 31(26) (2010) 6738-45.

[380] B.E. Uygun, A. Soto-Gutierrez, H. Yagi, M.L. Izamis, M.A. Guzzardi, C. Shulman, J. Milwid, N. Kobayashi, A. Tilles, F. Berthiaume, M. Hertl, Y. Nahmias, M.L. Yarmush, K. Uygun, Organ reengineering through development of a transplantable recellularized liver graft using decellularized liver matrix, Nat Med 16(7) (2010) 814-20.

[381] S.F. Badylak, The extracellular matrix as a biologic scaffold material, Biomaterials 28(25) (2007) 3587-93.

[382] J.L. Balestrini, L.E. Niklason, Extracellular Matrix as a Driver for Lung Regeneration, Ann Biomed Eng (2014).

[383] T.W. Hudson, S.Y. Liu, C.E. Schmidt, Engineering an improved acellular nerve graft via optimized chemical processing, Tissue Engineering 10(9-10) (2004) 1346-58.

[384] M. Ozeki, Y. Narita, H. Kagami, N. Ohmiya, A. Itoh, Y. Hirooka, Y. Niwa, M. Ueda, H. Goto, Evaluation of decellularized esophagus as a scaffold for cultured esophageal epithelial cells, J Biomed Mater Res A 79(4) (2006) 771-8.

[385] B. Yang, Y. Zhang, L. Zhou, Z. Sun, J. Zheng, Y. Chen, Y. Dai, Development of a porcine bladder acellular matrix with well-preserved extracellular bioactive factors for tissue engineering, Tissue Eng Part C Methods 16(5) (2010) 1201-11.

[386] L.E. Flynn, The use of decellularized adipose tissue to provide an inductive microenvironment for the adipogenic differentiation of human adipose-derived stem cells, Biomaterials 31(17) (2010) 4715-24.

[387] D. Wainwright, M. Madden, A. Luterman, J. Hunt, W. Monafo, D. Heimbach, R. Kagan, K. Sittig, A. Dimick, D. Herndon, Clinical evaluation of an acellular allograft dermal matrix in full-thickness burns, J Burn Care Rehabil 17(2) (1996) 124-36.

[388] L. Partington, N.J. Mordan, C. Mason, J.C. Knowles, H.W. Kim, M.W. Lowdell, M.A. Birchall, I.B. Wall, Biochemical changes caused by decellularization may compromise mechanical integrity of tracheal scaffolds, Acta Biomaterialia 9(2) (2013) 5251-61.

[389] S. Cebotari, I. Tudorache, T. Jaekel, A. Hilfiker, S. Dorfman, W. Ternes, A. Haverich, A. Lichtenberg, Detergent decellularization of heart valves for tissue engineering: toxicological effects of residual detergents on human endothelial cells, Artif Organs 34(3) (2010) 206-10.

[390] M.H. Zheng, J. Chen, Y. Kirilak, C. Willers, J. Xu, D. Wood, Porcine small intestine submucosa (SIS) is not an acellular collagenous matrix and contains porcine DNA: possible implications in human implantation, J Biomed Mater Res B Appl Biomater 73(1) (2005) 61-7.

[391] M.T. Kasimir, E. Rieder, G. Seebacher, E. Wolner, G. Weigel, P. Simon, Presence and elimination of the xenoantigen gal (alpha1, 3) gal in tissue-engineered heart valves, Tissue Engineering 11(7-8) (2005) 1274-80.

[392] T.J. Keane, R. Londono, N.J. Turner, S.F. Badylak, Consequences of ineffective decellularization of biologic scaffolds on the host response, Biomaterials 33(6) (2012) 1771-81.

[393] K.H. Yap, R. Murphy, M. Devbhandari, R. Venkateswaran, Aortic valve replacement: is porcine or bovine valve better?, Interact Cardiovasc Thorac Surg 16(3) (2013) 361-73.

[394] L.M. Wolford, D.B. Rodrigues, Autogenous grafts/allografts/conduits for bridging peripheral trigeminal nerve gaps, Atlas Oral Maxillofac Surg Clin North Am 19(1) (2011) 91-107.

[395] S. Letendre, G. LaPorta, E. O'Donnell, J. Dempsey, K. Leonard, Pilot trial of biovance collagen-based wound covering for diabetic ulcers, Adv Skin Wound Care 22(4) (2009) 161-6.

[396] S.P. Zhong, Y.Z. Zhang, C.T. Lim, Tissue scaffolds for skin wound healing and dermal reconstruction, Wiley Interdiscip Rev Nanomed Nanobiotechnol 2(5) (2010) 510-25.

[397] A. Khademhosseini, J.P. Vacanti, R. Langer, Progress in tissue engineering, Sci Am 300(5) (2009) 64-71.

[398] R. Parenteau-Bareil, R. Gauvin, F. Berthod, Collagen-Based Biomaterials for Tissue Engineering Applications, Materials 3(3) (2010) 1863-1887.

[399] N. Inoue, M. Bessho, M. Furuta, T. Kojima, S. Okuda, M. Hara, A novel collagen hydrogel cross-linked by gamma-ray irradiation in acidic pH conditions, J Biomater Sci Polym Ed 17(8) (2006) 837-58.

[400] A. Caporossi, C. Mazzotta, S. Baiocchi, T. Caporossi, Long-term results of riboflavin ultraviolet a corneal collagen cross-linking for keratoconus in Italy: the Siena eye cross study, Am J Ophthalmol 149(4) (2010) 585-93.

[401] K.S. Weadock, E.J. Miller, L.D. Bellincampi, J.P. Zawadsky, M.G. Dunn, Physical crosslinking of collagen fibers: comparison of ultraviolet irradiation and dehydrothermal treatment, Journal of Biomedical Materials Research 29(11) (1995) 1373-9.

[402] C.W. Yung, L.Q. Wu, J.A. Tullman, G.F. Payne, W.E. Bentley, T.A. Barbari, Transglutaminase crosslinked gelatin as a tissue engineering scaffold, J Biomed Mater Res A 83(4) (2007) 1039-46.

[403] M.J. van Luyn, P.B. van Wachem, L.H. Olde Damink, P.J. Dijkstra, J. Feijen, P. Nieuwenhuis, Secondary cytotoxicity of cross-linked dermal sheep collagens during repeated exposure to human fibroblasts, Biomaterials 13(14) (1992) 1017-24.

[404] I.V. Yannas, J.F. Burke, P.L. Gordon, C. Huang, R.H. Rubenstein, Design of an artificial skin. II. Control of chemical composition, Journal of Biomedical Materials Research 14(2) (1980) 107-32.

[405] H.M. Powell, S.T. Boyce, Wound closure with EDC cross-linked cultured skin substitutes grafted to athymic mice, Biomaterials 28(6) (2007) 1084-92.

[406] D.I. Zeugolis, G.R. Paul, G. Attenburrow, Cross-linking of extruded collagen fibers-A biomimetic three-dimensional scaffold for tissue engineering applications, Journal of Biomedical Materials Research 89A(4) (2009) 895-908.

[407] H.G. Sundararaghavan, G.A. Monteiro, N.A. Lapin, Y.J. Chabal, J.R. Miksan, D.I. Shreiber, Genipin-induced changes in collagen gels: correlation of mechanical properties to fluorescence, J Biomed Mater Res A 87(2) (2008) 308-20.

[408] M.J. van Luyn, P.B. van Wachem, L.O. Damink, P.J. Dijkstra, J. Feijen, P. Nieuwenhuis, Relations between in vitro cytotoxicity and crosslinked dermal sheep collagens, Journal of Biomedical Materials Research 26(8) (1992) 1091-110.

[409] E. Middelkoop, H.J. de Vries, L. Ruuls, V. Everts, C.H. Wildevuur, W. Westerhof, Adherence, proliferation and collagen turnover by human fibroblasts seeded into different types of collagen sponges, Cell Tissue Res 280(2) (1995) 447-53.

[410] FDA, Updated 510(k) Sterility Review Guidance K90-1;Guidance for Industry and FDA, in: D.o.H.H. Services (Ed.) FDA, 2002.

[411] S. Lerouge, A. Simmons, Sterilisation of biomaterials and medical devices, Elsevier2012.

[412] C.B.W. Group, Guidance for FDA Reviewers and Industry: Medical Devices Containing Materials Derived from Animal Sources (Except for In Vitro Diagnostic Devices). Nov. 6, 1998. US FDA, origin. www. fda. gov/cdrh/ode/88. html.

[413] E.M. Agency, Minimising the risk of transmitting animal spongiform encephalopathy agents via human and veterinary medicinal products. EMA/410/01 rev. 3. < https://www.edqm.eu/site/General_chapter_528_Minimising_the_risk_of_transmitting_animal_spongiform_encephalopathy_agents_via_human_and_veterinary_medicinal_products-en-30598-2.html.>, 2011 (accessed 27 October.2014).

[414] G.C. Mendes, T.R. Brandao, C.L. Silva, Ethylene oxide sterilization of medical devices: a review, American journal of infection control 35(9) (2007) 574-81.

[415] A.M. Matuska, P.S. McFetridge, The effect of terminal sterilization on structural and biophysical properties of a decellularized collagen-based scaffold; implications for stem cell adhesion, Journal of biomedical materials research. Part B, Applied biomaterials (2014).

[416] L.H.H.O. Damink, P.J. Dijkstra, M.J.A. Vanluyn, P.B. Vanwachem, P. Nieuwenhuis, J. Feijen, Influence of Ethylene-Oxide Gas Treatment on the in-Vitro Degradation Behavior of Dermal Sheep Collagen, Journal of biomedical materials research 29(2) (1995) 149-155.

[417] K.A. Faraj, K.M. Brouwer, P.J. Geutjes, E.M. Versteeg, R.G. Wismans, J.A. Deprest, H. Chajra, D.M. Tiemessen, W.F.J. Feitz, E. Oosterwijk, W.F. Daamen, T.H. Kuppevelt, The Effect of Ethylene Oxide Sterilisation, Beta Irradiation and Gamma Irradiation on Collagen Fibril-Based Scaffolds, Tissue Eng Regen Med 8(5) (2011) 460-470.

[418] W.A. Rutala, D.J. Weber, Disinfection of endoscopes: review of new chemical sterilants used for high-level disinfection, Infection control and hospital epidemiology : the official journal of the Society of Hospital Epidemiologists of America 20(1) (1999) 69-76.

[419] S. Yoganarasimha, W.R. Trahan, A.M. Best, G.L. Bowlin, T.O. Kitten, P.C. Moon, P.A. Madurantakam, Peracetic acid: a practical agent for sterilizing heat-labile polymeric tissue-engineering scaffolds, Tissue engineering. Part C, Methods 20(9) (2014) 714-23.

[420] W.A. Rutala, Weber, D.J. , Guideline for Disinfection and Sterilization in Healthcare Facilities, in: C.f.D.C.a. Prevention (Ed.) Atlanta, 2008, p. 64.

[421] M. Markowicz, E. Koellensperger, G.C. Steffens, M. Frentz, N. Schrage, N. Pallua, The impact of vacuum freeze-drying on collagen sponges after gas plasma sterilization, Journal of biomaterials science. Polymer edition 17(1-2) (2006) 61-75.

[422] A.D. Lucas, K. Merritt, V.M. Hitchins, T.O. Woods, S.G. McNamee, D.B. Lyle, S.A. Brown, Residual ethylene oxide in medical devices and device material, J Biomed Mater Res B Appl Biomater 66(2) (2003) 548-52.

[423] A.M. Sikin, S.S. Rizvi, Recent patents on the sterilization of food and biomaterials by supercritical fluids, Recent patents on food, nutrition & agriculture 3(3) (2011) 212-25.

[424] F.A. Herdegen V., Haseneder R., Repke JU., Leppchen-Fröhlich K., Prade I., Meyer M., Sterilization of Medical Products from Collagen by Means of Supercritical CO2, Chem. Eng. Technol. 37(7) (2014) 6.

[425] M. Moisan, J. Barbeau, S. Moreau, J. Pelletier, M. Tabrizian, L.H. Yahia, Low-temperature sterilization using gas plasmas: a review of the experiments and an analysis of the inactivation mechanisms, International Journal of Pharmaceutics 226(1–2) (2001) 1-21.

[426] H. Shintani, A. Sakudo, P. Burke, G. McDonnell, Gas plasma sterilization of microorganisms and mechanisms of action, Experimental and therapeutic medicine 1(5) (2010) 731-738.

[427] M.C. Krebs, P. Bécasse, D. Verjat, J.C. Darbord, Gas–plasma sterilization: relative efficacy of the hydrogen peroxide phase compared with that of the plasma phase, International Journal of Pharmaceutics 160(1) (1998) 75-81.

[428] D.T. Cheung, N. Perelman, D. Tong, M.E. Nimni, The effect of gamma-irradiation on collagen molecules, isolated alpha-chains, and crosslinked native fibers, Journal of biomedical materials research 24(5) (1990) 581-9.

[429] A.J. Bailey, W.J. Tromans, Effects of Ionizing Radiation on the Ultrastructure of Collagen Fibrils, Radiation research 23 (1964) 145-55.

[430] M.P. Ohan, M.G. Dunn, Glucose stabilizes collagen sterilized with gamma irradiation, Journal of biomedical materials research. Part A 67(4) (2003) 1188-95.

[431] R.S. Benson, Use of radiation in biomaterials science, Nucl Instrum Meth B 191 (2002) 752-757.

[432] P. Dethier, Device Sterilisation: The X Factor, European Medical Device Technology 2(5) (2011).

[433] Y. Shamis, S. Patel, A. Taube, Y. Morsi, I. Sbarski, Y. Shramkov, R.J. Croft, R.J. Crawford, E.P. Ivanova, A New Sterilization Technique of Bovine Pericardial Biomaterial Using Microwave Radiation, Tissue Eng Part C-Me 15(3) (2009) 445-454.

[434] K.D. Andrews, J.A. Hunt, R.A. Black, Effects of sterilisation method on surface topography and in-vitro cell behaviour of electrostatically spun scaffolds, Biomaterials 28(6) (2007) 1014-1026.

[435] Y. Kawasaki, S. Sotome, T. Yoshii, I. Torigoe, H. Maehara, Y. Sugata, M. Hirano, N. Mochizuki, K. Shinomiya, A. Okawa, Effects of gamma-ray irradiation on mechanical properties, osteoconductivity, and absorption of porous hydroxyapatite/collagen, J Biomed Mater Res B Appl Biomater 92(1) (2010) 161-7.

[436] K. Duprez, J. Bilweis, A. Duprez, M. Merle, Experimental and clinical study of fast absorption cutaneous suture material, Annales de chirurgie de la main : organe officiel des societes de chirurgie de la main 7(1) (1988) 91-6.

[437] C.F. van Eck, A.F. Chen, B.A. Klatt, J. D'Antonio, F. Fu, The classification of implants: class I, II, III, Journal of long-term effects of medical implants 19(3) (2009) 185-93.

[438] J. Bergsland, O.J. Elle, E. Fosse, Barriers to medical device innovation, Medical devices (Auckland, N.Z.) 7 (2014) 205-9.

[439] A. F2027-08, Standard Guide for Characterization and Testing of Raw or Starting Biomaterials for Tissue-engineered Medical Products, ASTM International (2008).

[440] P. Van Wilder, Advanced therapy medicinal products and exemptions to the Regulation 1394/2007: how confident can we be? An exploratory analysis, Frontiers in pharmacology 3 (2012).

[441] I. Vinck, F. Hulstaert, H. Van Brabandt, M. Neyt, S. Stordeur, Market introduction of innovative high risk medical devices: towards a recast of the directive concerning medical devices, European journal of health law 18(5) (2011) 477-89.

TISSUE ENGINEERING OF THE URETHRA:

A systematic review and meta-analysis of pre-clinical and clinical studies

Luuk R. Versteegden*
Paul K.J.D. de Jonge*
Joanna IntHout
Toin H. van Kuppevelt
Egbert Oosterwijk
Wout F. Feitz
Rob B.M. de Vries
Willeke F. Daamen
*Contributed equally

European Urology. OCT 2017, volume 72, issue 4, p594-606.

Abstract

Urethra repair by tissue engineering has been extensively studied in laboratory animals and patients, but is not routinely used in clinical practice. The objective of this review was to systematically investigate pre-clinical and clinical evidence of the efficacy of tissue engineering for urethra repair in order to stimulate translation of pre-clinical studies to the clinic.

A systematic search strategy was applied in PubMed and EMBASE. Studies were independently screened for relevance by two reviewers, resulting in 80 pre-clinical and 23 clinical studies of which 63 and 13 were selected for meta-analysis to assess side-effects, functionality, and study completion. Analyses for pre-clinical and clinical studies were performed separately. Full circumferential and inlay procedures were assessed independently. Evaluated parameters included seeding of cells and type of biomaterial.

Meta-analysis revealed that cell seeding significantly reduced the probability of encountering side-effects in pre-clinical studies. Remarkably though, cells were only sparsely used in the clinic (4/23 studies) and showed no significant reduction of side-effects. ln 21 out of 23 clinical studies, decellularized templates were used, while in pre-clinical studies other biomaterials showed promising outcomes as well. No direct comparison to current clinical practice could be made due to the limited number of randomized controlled studies.

Due to a lack of controlled (pre-)clinical studies, the efficacy of tissue engineering for urethra repair could not be determined. Meta-analysis outcome measures were similar to current treatment options described in literature. Surprisingly, it appeared that favorable pre-clinical results, i.e. inclusion of cells, were not translated to the clinic. Improved (pre-)clinical study designs may enhance clinical translation.

Patient Summary: We reviewed all available literature on urethral tissue engineering to assess the efficacy in pre-clinical and clinical studies. We show that improvements to (pre-)clinical study design is required to improve clinical translation of tissue engineering technologies.

1. Introduction

Congenital birth defects of the urethra, such as hypospadias (1 in every 300 births) [1, 2], and acquired urethral abnormalities, such as urethral strictures (1 in every 1,000 men >65 years of age [3]), represent major clinical entities. Treatment usually involves a surgical procedure with risk of (recurrence of) strictures or fistula requiring additional care or reintervention. Whenever possible, local tissue flaps or stricture resection in combination with end-to-end anastomosis are used for urethra reconstruction [4, 5]. Generally, two surgical approaches exist for urethral reconstruction: partial replacements using onlay or inlay techniques or the full circumferential procedure, which is used in rare cases with significant urethral scarring or lichen sclerosis. Depending on patient and local factors, procedures can be performed as one-stage procedure or as planned multistage procedure [3]. Autologous tissue transplantation such as buccal mucosa or free skin grafts are the standard treatments [6-9]. However, due to the limited quantity of available donor tissue, accompanying donor site morbidity (16 to 32% for buccal mucosa grafts) and complications (*e.g.* recurrences or infections), alternative treatment options are needed to improve long-term outcome [10]. Tissue engineering may overcome some of the aforementioned disadvantages by providing a temporary template to guide tissue regeneration [11]. In general, tissue engineered templates include decellularized tissue or *de-novo* prepared materials from natural or synthetic origin [12-14]. Templates can be seeded with (stem) cells from the patient prior to implantation. These cells may stimulate tissue remodeling by excreting cytokines and growth factors and contributing to cellular population of the template [15, 16].

Despite the potential of tissue engineering shown in *in vitro* research and pre-clinical studies, clinical translation is limited. To improve translation, an evidence-based approach, such as systematic reviews, can be applied when designing new tissue engineering strategies. This will avoid unnecessary replication of studies and will help to select the most optimal experimental design and model. We are the first to perform a comprehensive systematic review of evidence for the efficacy of urethral tissue engineering in pre-clinical and clinical studies. A meta-analysis was used to compare different experimental designs based on clinically relevant outcomes. This systematic review aims to improve the translation of urethral tissue engineering from bench to bedside.

2. Evidence acquisition

2.1. Literature search

To identify all available studies on urethral tissue engineering published and indexed up until June 1, 2016, a systematic search strategy was applied in PubMed (Appendix 1) and Embase (via OvidSP; Appendix 2). This strategy combined a tissue engineering search component containing synonyms for tissue engineering related terms [17] with a customized search component for urethra or urethra-related diseases. MeSH terms and EMTREE terms were used in PubMed and Embase, respectively, together with separate words or word combinations in title or abstract. Next, either an animal filter designed by Hooijmans *et al.* (PubMed) [18] or de Vries *et al.* (Embase) [19] was applied (Appendix 1 and 2, search component 3A) or a custom filter for clinical studies (Appendix 1 and 2, search component 3B). In addition, retrieved reviews were screened for primary studies not found using the search strategy. Clinical studies found during animal search strategy were marked and screened for relevance and *vice versa*.

2.2. Study selection

Duplicates in retrieved articles were removed in EndNote (Version X7.2, Thomson Reuters). Studies were assessed independently by LV and PdJ. First, clearly irrelevant studies were excluded based on title. Next, titles and abstracts of the remaining articles were screened for relevance in Early Review Organizing Software (EROS, Buenos Aires, Argentina, www.eros-systematic-review.org) using the following exclusion criteria: 1) no urethra, 2) no tissue engineering, 3) no animals or patient, 4) no primary study. A study was considered to be about tissue engineering when a processed template was used. Studies on tissue transplants or reconstructive surgery without the use of a template or without a urethra defect were excluded. Of the remaining studies full texts were screened using the same exclusion criteria. Articles not available as full text were excluded at this stage. No language restrictions were applied in the screening phase. If necessary, Google translate was used. Retrieved studies from search updates were directly screened in Endnote according to the same principles. In all stages of the selection process, discrepancies between reviewers were discussed until consensus was reached.

2.3. Study characteristics

From all included studies, general information (author, year) and study characteristics (age range of patients, animal species, sex, surgical procedure, type of biomaterial, type of cells) were extracted and listed in Table 1 for pre-clinical studies and Table

2 for clinical studies. For languages other than English, German and French, Google Translate was used to retrieve study characteristics.

2.4. Extraction outcome data

Three outcome measures were used to evaluate study outcome: 1) incidence of side effects, *e.g.* strictures, stenosis, fistulae, and infections, 2) functionality, defined as the ability to void with continence, and 3) study completion, for animals defined as survival until predetermined endpoint and for clinical studies as available for follow-up or no additional urethroplasty required. Only English, German and French studies were considered for quality assessment and meta-analysis. When critical information needed (e.g. surgical procedure or number of animals/patients) was incomplete, studies were excluded. As only two studies used rats these were also excluded at this stage.

2.5. Quality assessment

Due to the non-randomized, non-controlled nature of most pre-clinical and clinical studies, no standard risk of bias analysis could be performed as validated tools are unavailable for these types of studies. Instead, overall quality was independently scored by PdJ and LV based on the reporting of specific key information (Figure 2, Results section). Discrepancies were discussed until agreement was reached.

2.6. Meta-analysis

The following main research question was considered: “What is the evidence for the efficacy of urethral tissue engineeing in pre-clinical and clinical studies?” Sub-questions included the effects of the addition of (stem) cells to the template, the type of biomaterial, as well as potential differences between animal species on the separate outcome measures. Analyses for pre-clinical and clinical studies were conducted separately, as were full circumferential and inlay procedures. Statistical analyses were performed with SAS/STAT® software v9.2 for Windows, copyright© 2002-2008 by SAS Institute Inc., Cary, NC, USA.

2.6.1. Pre-clinical studies

The following pre-clinical data were extracted for all available time points per study: the total number of animals as well as the number of animals without side effects, with functionality, and alive at the study endpoint. Time points were categorized in three periods: 0-4 weeks, 5-11 weeks and 12 weeks or longer.

Per study, the probability of response (*e.g.* having no side effects) with a corresponding 95% exact (Clopper-Pearson) confidence interval (CI) was estimated per outcome. An additive random-effects logistic meta-regression model was fitted by means of a generalized linear mixed model approach. The number of responding animals out of the total was used as outcome parameter. In addition, the following independent parameters were used: treatment (combining the addition of cells and the type of biomaterial) and animal species. Random effects for study and for treatment grouped by study, were added. The Akaike Information Criterion [20] showed that models based on combined study data were preferable to models based on the period data (period as factor), therefore all time points per study were combined. When possible, the maximum likelihood approach with adaptive quadrature was used as estimation method. If this did not converge, the maximum likelihood with the Laplace approximation was applied. The resulting estimated odds were backtransformed into percentages and corresponding 95% CIs. In addition, the marginal effects of the treatments were estimated by combining the estimated percentages for rabbits and dogs, including 95% logit-based CIs, as described by Zou [21]. P-values were based on these confidence intervals.

2.6.2. Clinical studies

For the analyses of the clinical outcomes, the following data per study were extracted: total number of patients, and numbers of patients without side effects, with functionality, and completing the study. No separate time points were analyzed in the human studies. For each study, the probability of response with corresponding 95% exact CIs was estimated per outcome. Due to limited study diversity, meta-regression models similar to pre-clinical studies were only fit for inlay repair and biomaterial type 'decellularized'. A compound symmetry random effect was added for the addition of cells, grouped by study. Estimated odds from meta-regression were backtransformed into probabilities and corresponding 95% CIs.

3. Evidence synthesis

3.1. Literature search and screening

Figure 1A and B show the results of the literature search and screening of collected studies. After the search, 1,524 unique pre-clinical and 5,361 unique clinical studies were identified. During title and abstract screening of these studies, 1,349 and 5,282 were excluded, respectively. After full text screening, 80 pre-clinical studies and 23

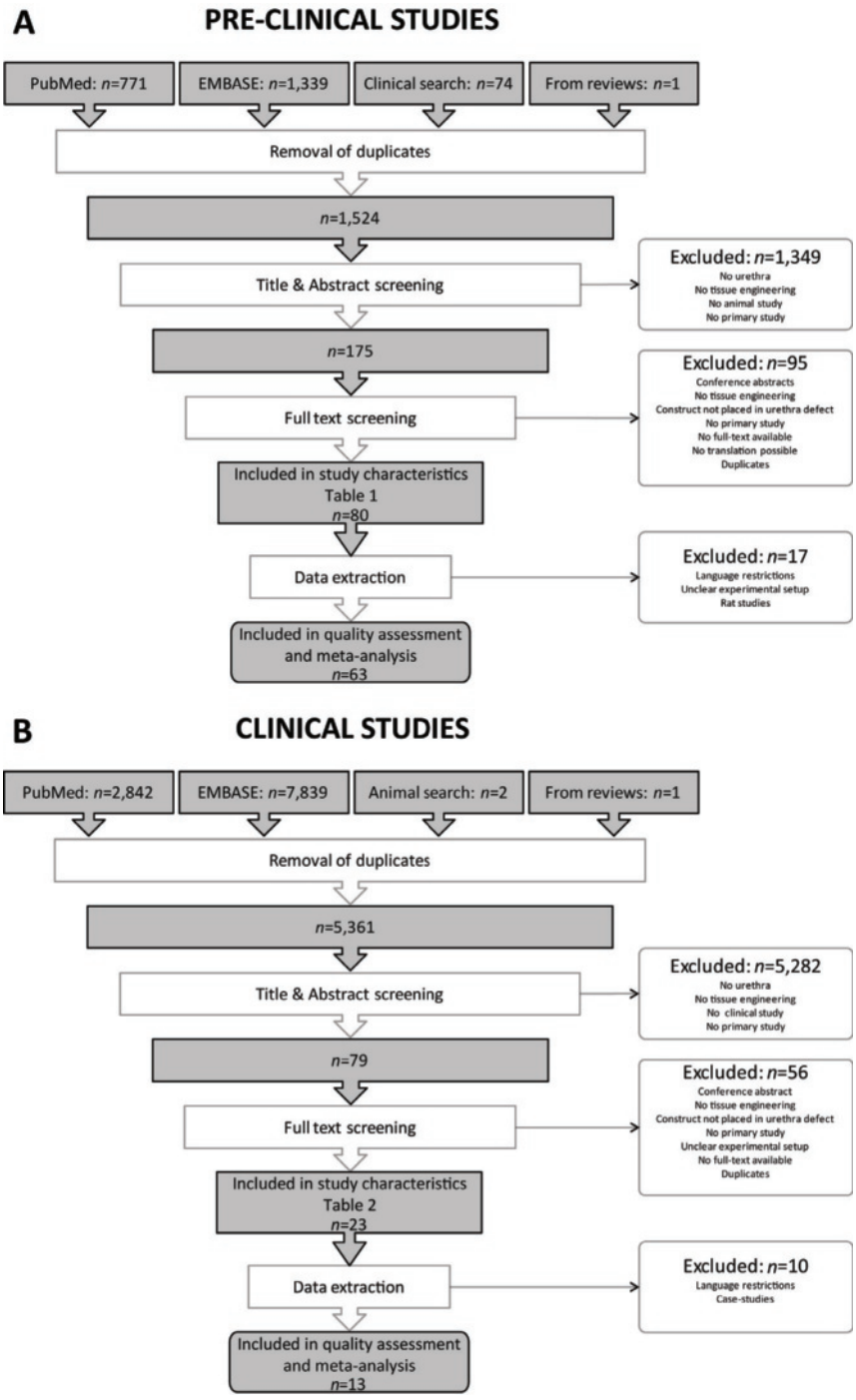


Figure 1. Flowchart of search and screening process of A) pre-clinical studies and B) clinical studies. The search was updated until June 1, 2016.

clinical studies were included in the study characteristics table (see section 3.2). Only 63 pre-clinical and 13 clinical studies were eligible for the quality assessment (section 3.3) and meta-analysis (section 3.4).

3.2. Study characteristics

3.2.1. Pre-clinical studies

Pre-clinical study characteristics are summarized in Table 1 at the end of this chapter (see Appendix 3 for references of listed studies). Only three animal species, rabbits (59/80), dogs (19/80) and rats (2/80) were used, which were predominantly males (72/80). Full circumferential repair was investigated in 41 studies, inlay repair in 30 studies, both methods in three, while the procedure was unclear in the remaining studies (6/80). In dogs, primarily full defect repairs were performed (14 full vs. 4 inlay), while in rabbits both inlay (25) and full repairs (26) were employed.

Due to the wide variety of materials used, they were categorized into three categories: decellularized templates (46/80), *de novo* prepared templates from natural materials (18/80), and *de novo* prepared templates from synthetic materials (12/80). Four (4/80) studies used multiple material types in different groups and these were assessed separately in the meta-analysis. Synthetic materials were almost exclusively used for full repair (10 full vs. 3 inlay). Cells were incorporated into templates in 34 studies, of which bladder smooth muscle cells (SMCs) and urothelial cells were mostly used (13/34), followed by keratinocytes and fibroblasts from oral tissue (6/34) or a combination thereof (2/34), foreskin epidermal cells (2/34) and omental mesothelial cells (1/34). Stem cells, mostly derived from adipose tissue, bone marrow or human umbilical cord, were used in 10 studies.

3.2.2. Clinical studies

Study characteristics of clinical studies are listed in Table 2 at the end of this chapter (see Appendix 4 for references of listed studies). Clinical studies were performed with males, except for one study (Table 2, #22). From 23 studies, 16 used an inlay approach, two a full circumferential procedure, one used both approaches, while in four studies the procedure was unclear. The majority of studies (21/23) used decellularized templates, while natural and synthetic templates were both used once. Four studies used cell-seeded templates; 2/23 buccal mucosa keratinocytes and/or fibroblasts and 2/23 bladder SMCs and/or urothelial cells.

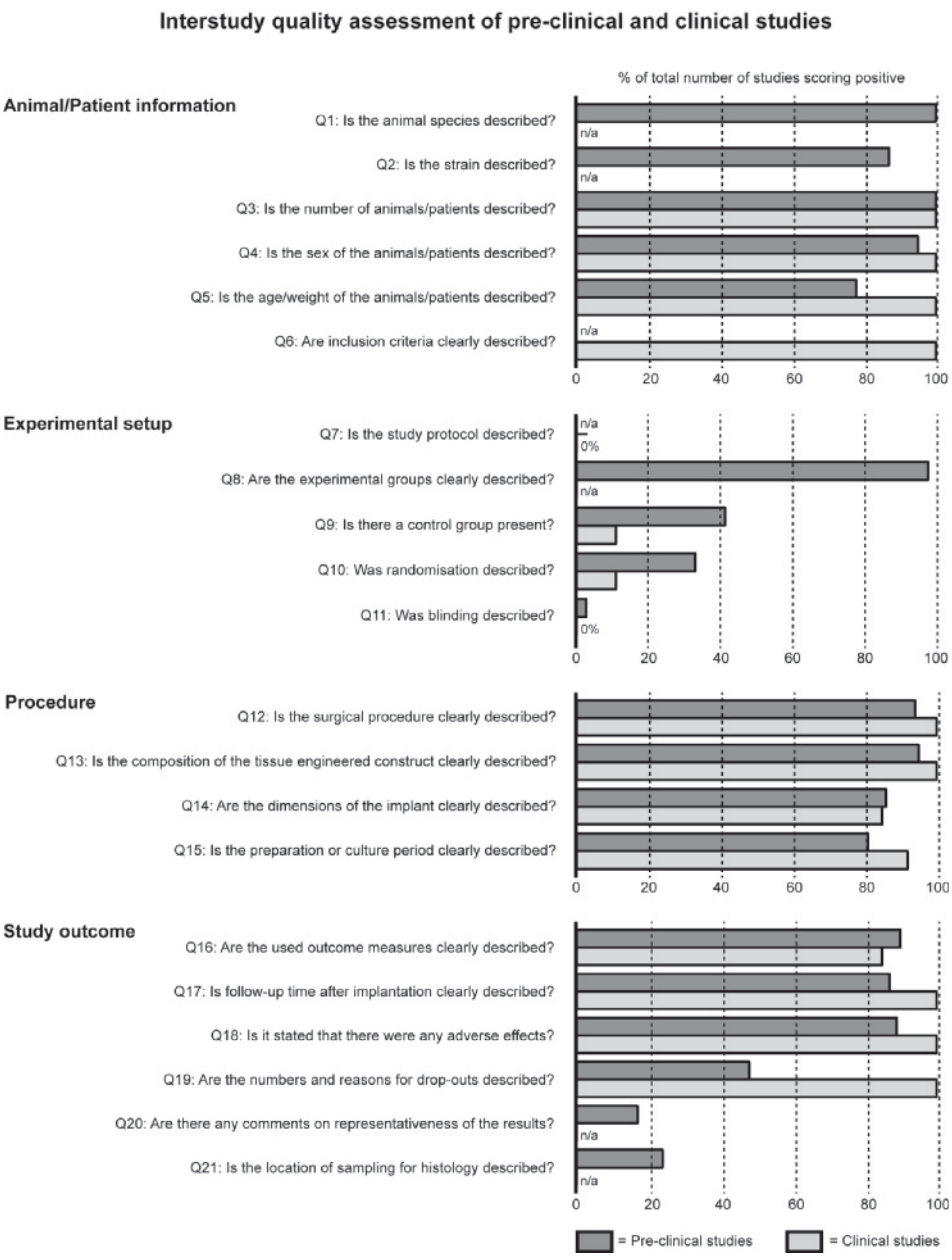


Figure 2. Quality assessment of pre-clinical and clinical studies. All studies included for meta-analysis were scored on clear reporting of several key parameters (Q1-Q21) showing that study design such as inclusion of proper control groups, associated randomization and blinding, reporting of key parameters such as representativeness of shown results and drop-outs needs to be improved in pre-clinical studies.

3.3. Quality assessment

The quality of reporting was assessed for 63 pre-clinical and 13 clinical studies from which outcome data could sufficiently be extracted for inclusion in the meta-analysis (Figure 2). Results per study are listed in Appendix 5. Reporting of information regarding included animals/patients, such as species and strain, sex, number of animals/patients, age/weight and patient inclusion criteria, were generally well described.

Overall quality of the experimental setup was poor. Although the different experimental groups were well described, hardly any control groups were present, and randomization and blinding were seldomly mentioned in both pre-clinical and clinical studies. Also, clinical study protocols were not published. However, surgical procedure, composition, size and preparation of the implants were clearly described in most studies. Reporting of outcome measures was good for both pre-clinical and clinical studies with respect to the description of outcome measures, follow-up time and side effects. The number of drop-outs was clearly mentioned in clinical studies, but only in half of the pre-clinical studies. For pre-clinical studies, histological sampling location and representativeness of the results were poorly described.

3.4. Meta-analysis

3.4.1. Pre-clinical studies

For full circumferential repair (Figure 3A), the addition of cells significantly reduced the probability of side effects, independent of the type of biomaterial used (p=0.001). Exact point estimates including CI are given in Appendix 6. Regarding the type of biomaterial, when no cells were used, estimates show that synthetic materials had a higher probability for having no side-effects compared to decellularized and natural materials. With cells seeded, estimated probabilities were similar for all materials. For functionality and study completion, estimated probabilities were similar for all study conditions.

For inlay repair (Figure 3B), the addition of cells significantly reduced the probability of side effects (p=0.003), albeit less than for full repair. Estimated probabilities were similar for all types of biomaterial regardless of the addition of cells. For functionality and study completion, estimated probabilities were similar for all study conditions. It was impossible to estimate study completion probability per biomaterial as almost all animals survived inlay repair (statistical model did not converge).

Although estimated probabilities for dogs and rabbits were slightly different, differences were not statistically significant. Consequently, the animal species had only marginal influence on outcome (data not shown).

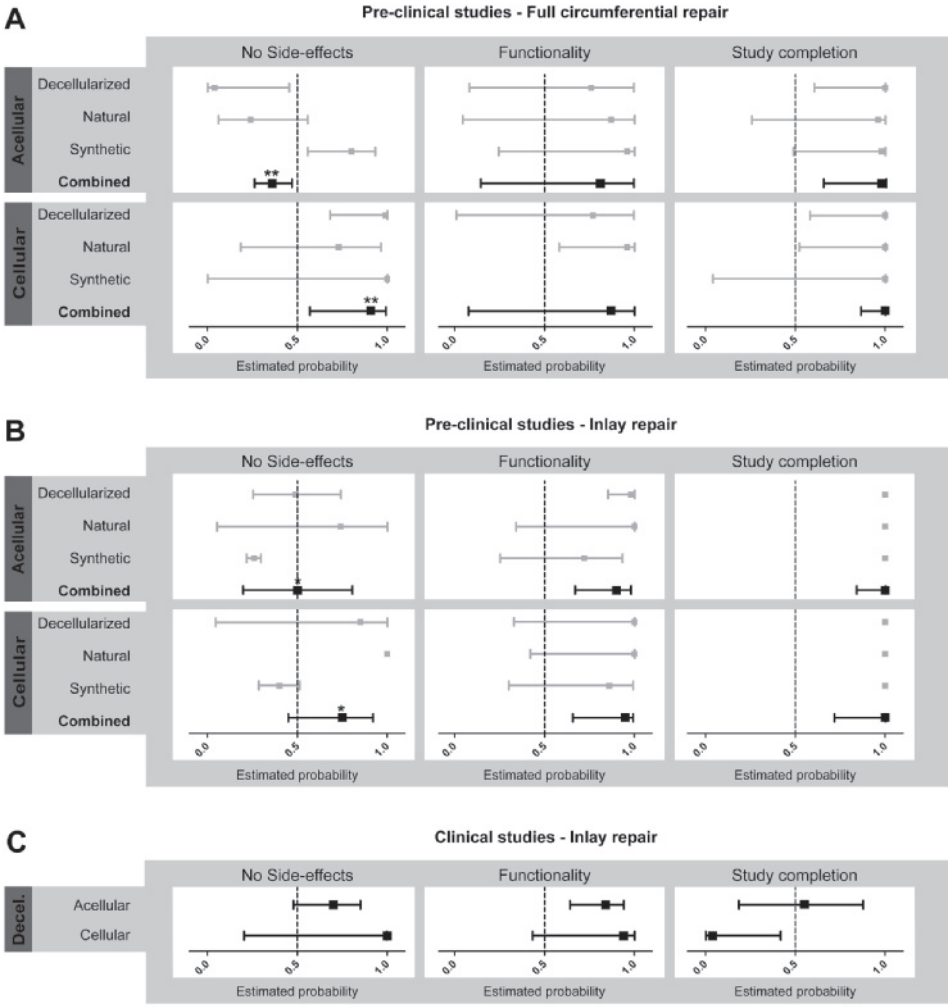


Figure 3. Estimated probability including 95% confidence intervals for the absence of side effects, functionality and study completion for A) full circumferential repair and B) inlay repair in pre-clinical studies, both categorized for the use of cells and the type of biomaterial. C) For the clinical studies, only decellularized material with or without cells could be analyzed. The effect of cells on the three outcome measures was calculated in estimated probabilities. Overall differences for cellular vs. acellular templates were determined for each outcome measure for both full and inlay repair: * p=0.003, ** p=0.001, all other differences were not significant (p>0.05). Specific point estimates and confidence interval are given in Appendix 6.

3.4.2. Clinical studies

For clinical studies, a similar meta-analysis was performed (Figure 3C). Only inlay repair using decellularized materials with or without cells could be analyzed due to the limited number of other combinations. No statistically significant differences were found for the inclusion of cells for any of the outcome measures effects, p=0.7 for functionality (p=0.5 for side- and p=0.08 for study completion).

When comparing pre-clinical and clinical estimated probabilities, point estimates for absence of side-effects after inlay repair seem to be higher in clinical studies for both acellular and cellular templates. For functionality, the point estimates were similar. The estimated probability for study completion was much lower in clinical studies compared to pre-clinical studies regardless of the addition of cells, but these cannot be directly compared due to distinctive definitions for study completion and differences in disease status.

4. Discussion

Reconstructive surgery using biomaterials has been studied as an alternative approach for urethral repair since the early seventies and efforts along these lines expanded rapidly in the nineties when the term 'Tissue Engineering' was introduced (Figure 4) [11]. Nowadays, pre-clinical studies have been readily performed, but clinical studies have not followed this trend. Although many (pre-)clinical studies have been

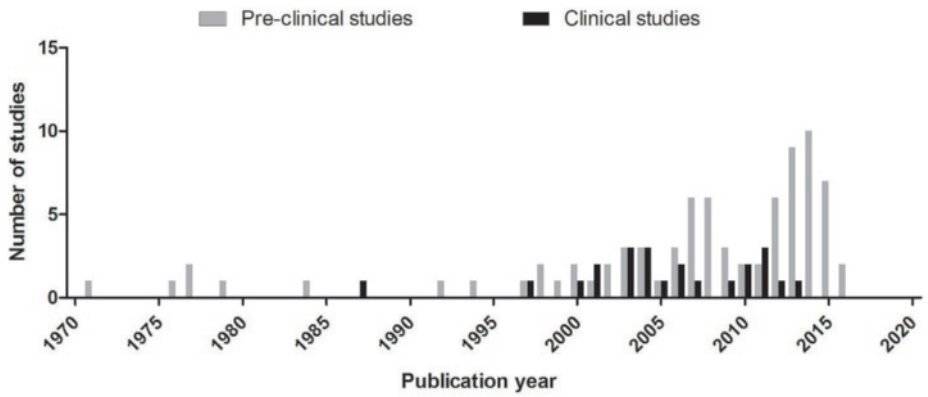


Figure 4. Number of publications per year for pre-clinical and clinical studies included in this systematic review. After several single studies between 1971 and 1994, the number of publications increased. Peaks in both clinical and pre-clinical studies were seen around 2005-2008 and again between 2012-2015.

performed, tissue engineering is not used as an alternative treatment in routine clinical practice, except for a select patient group with a history of failed repairs [22-24]. In this systematic review, all (pre-)clinical publications on urethra tissue engineering until June 2016 were analyzed to assess the evidence for the efficacy. For clinical studies, the term "effectiveness" may be more suitable, as most studies included in this review showed a heterogeneous patient population [25]. However, we used the term "efficacy" for pre-clinical and clinical studies throughout this systematic review. For both pre-clinical and clinical studies, tissue engineering had a high probability for functionality, defined as voiding with continence. Study completion was high in pre-clinical studies, but not in clinical studies. This may be related to the difference in our definition of study completion and in study design. In pre-clinical studies, animals generally only need to survive for several months to study the tissue regeneration process, compared to patients that need to show a good long term outcome without reintervention and without being lost in follow-up. Most patients had a history of failed repairs using conventional techniques, while healthy animals were used. As randomized clinical studies were lacking, *e.g.* comparison with standard treatments (free skin graft or buccal mucosa urethroplasty) [3], no direct comparisons with current clinical practice could be made. Available literature about complex two-stage urethroplasty shows complication-free rates, functionality and study completion of approximately 62%, 67% and 36% [26], similar to the outcome of tissue engineered urethras (based on point estimates). This suggests that tissue engineered urethras may perform adequately and may be a valid alternative. Clearly, randomized controlled clinical trials are needed to clarify this issue.

Application of cells

There is no consensus on the potential beneficial effects of cell seeding of tissue engineered constructs for the urogenital system. For tissue engineering of the bladder, the addition of cells did not give an overall beneficial effect on tissue regeneration [27], while others claim that cells are required for urethra repair of constructs >0.5 cm [28]. For urethra tissue engineering, the inclusion of cells significantly reduced side effects in pre-clinical studies for both full ($p=0.001$) and inlay ($p=0.003$) defects. In other, less comprehensive systematic reviews, a similar outcome regarding the effectiveness of the addition of cells was shown [29, 30]. For full defects, cell addition has more added value, which may be explained by the fact that cells can only infiltrate from the two urethra edges, while in inlay repair cell ingrowth can also occur from the sides, boosting cell coverage.

The effects of cell addition on functionality and study completion were not significant, regardless of surgical procedure. This may be caused by the short follow-up period underestimating long-term complications, such as complete strictures. Meta-analysis of clinical studies showed no significant effect of cells for any of the outcome measures. Consequently, the use of cells for the repair of urethra in the clinic remains debatable.

Type of biomaterial

Meta-analysis showed no differences in estimated probabilities for the different materials in most of the conditions, with the exception of synthetic materials showing better estimated probabilities than natural materials in full circumferential repair without cells regarding side-effects. For inlay repair in pre-clinical studies, synthetic materials did not perform as well as in full repair, but only a limited number of studies was reported.

Decellularized materials were used in the vast majority of clinical studies. This may be related to the experience with decellularized materials in other fields of tissue engineering, such as skin tissue engineering [31]. Which type of biomaterial is superior to the current state-of-the-art remains to be established.

Selection of animal species

The choice of animal species is often based on financial issues, experience of the researchers, ethical arguments and practical restrictions [32-34]. An evidence-based approach can aid in selection of the most appropriate model. In this review, differences between treatment were not notably influenced by the choice for rabbit or dog, however a higher statistical power would strengthen this claim.

Clinical relevance and limitations of pre-clinical and clinical studies

Quality of the experimental designs and reporting of pre-clinical studies was generally low. Proper control groups, such as sham operation groups and standard treatment groups, were often lacking. Instead, the experimental material without cells was generally considered the control. In addition, outcome measures and drop-outs were not specifically reported for each animal, complicating data interpretation. Also, representativeness of presented data was often not mentioned. This may have hampered clinical translation of these pre-clinical findings. To improve this, all design parameters and outcomes should be specifically documented for individual animals similar to patients in clinical studies. The “Gold standard publication checklist to

improve the quality of animal studies" by Hooijmans *et al.* would be helpful for the design and reporting of pre-clinical studies [35].

Another limitation for the level of evidence provided by the pre-clinical studies is the use of healthy animals, in which a created defect is immediately closed, compared to patients with a history of stricture, lichen sclerosis or hypospadias. From the patients in clinical studies 75% had one or more previous treatments, e.g. dilation, urethrotomy or urethroplasty, before attempting the tissue engineered constructs. The requirement of animal models with injury or disease has been shown in other fields [36] and should also be considered in tissue engineering, in this particular situation by inducing strictures.

Clinical studies provided a low level of evidence due to their setup, making the true effect of tissue engineering as surrogate for the current standard treatment unclear. Only El-Kassaby *et al.* (Table 2, #3) performed a small randomized controlled study. To improve the level of evidence, more randomized controlled studies are needed, preferably with larger numbers of patients and longer follow-up. Compared to the pre-clinical studies, reporting of important parameters was much better, notably regarding drop-outs and adverse events. Nevertheless, to further improve the quality of the clinical studies, the study protocol should be published with the manuscript and a detailed description of patient inclusion criteria (e.g. sex, age and medical history) should be provided.

The level of evidence is further limited by original research's susceptibility to publication bias [37], which may lead to overestimation of the treatment effect in pre-clinical studies. Recognition of this bias may partly explain the poor translation of tissue engineering techniques to the clinic.

Furthermore, pre-clinical studies should better support the clinical need: the majority of pre-clinical studies involves full circumferential repair, where clinicians mainly perform inlay repair [3]. This may be explained by pre-clinical researchers attempting to prove the effectiveness of the experimental treatment for the most problematic (circumferential) procedures, assuming that it will also be effective in less complicated (inlay) approaches.

Finally, inclusion of cells remains challenging in a clinical setting as no beneficial effect was seen (in 11 patients), even though this significantly improved pre-clinical

outcome. It is possible that inclusion of cells was perceived as too problematic, despite better results in a pre-clinical setting and that in the final assessment the choice was driven by parameters other then pre-clinical outcome. To consider cells for clinical applications, its efficacy has to be proven as the use of cells involves extensive regulatory requirements which may hamper clinical application [38-40]. In addition, the costs of cellular implants will be higher compared to off-the-shelf acellular implants, since two procedures are needed (cell harvesting in urine or biopsy, and urethroplasty) and *in vitro* cell expansion may be needed [41, 42].

5. Conclusions

The efficacy of tissue engineering for urethra repair could not be determined due to a lack of controlled (pre-)clinical studies. However, meta-analysis outcomes (side-effects, functionality and study completion) were comparable to current treatment options described in literature, indicating the potential of tissue engineering for urethra repair. The findings of this systematic review may result in improved study design which may aid the translation of tissue engineered urethras to the clinic as an alternative for autografts.

6. Acknowledgements

This study was financially supported by PIDON (NovioTissue project), a subsidiary program of the Dutch Ministry of Economic Affairs and the states of Gelderland and Overijssel (PID101020).

7. Supplemental Information

Supplemental information is available at https://doi.org/10.1016/j.eururo.2017.03.026.

Table 1: Study characteristics of all 80 pre-clinical studies sorted on inclusion in meta-analysis, surgical procedure and biomaterial.

	Author	Year	Animal model	# of animals	Sex	Surgical Procedure (defect length in mm)	Biomaterial (category)	Type of added cells	Quality Assessment + Meta-analysis
1	Feng, C.	2011	Rabbit	28	M	Inlay (15)	Acellular corpus spongiosum, porcine (D)	Autologous corporal SMC's and lingual keratinocytes	Yes
2	Ayyildiz, A.	2006	Rabbit	10	M	Inlay (5)	Alloderm® + acellular pericardium, bovine (D)	-	Yes
3	Chen, F.	1999	Rabbit	10	M	Inlay (10)	BAM, porcine (D)	-	Yes
4	Chun, S.Y.	2015	Rabbit	10	M	Inlay (20)	BAM, porcine (D)	Autologous minced urethral muscle and urothelial tissue	Yes
5	Sayeg, K.	2013	Rabbit	18	M	Inlay (35)	BAM, porcine (D)	Autologous bladder SMC's	Yes
6	Huang, J.W.	2014	Rabbit	30	M	Inlay (15)	BAM, rabbit (D)	-	Yes
7	Li, C.	2008	Rabbit	24	M	Inlay (20)	BAM, rabbit (D)	Autologous oral keratinocytes	Yes
8	Li, C.	2013	Rabbit	27	M	Inlay (20)	BAM, rabbit (D)	Autologous oral kereatincytes and TGF-β siRNA transfected fibroblasts	Yes
9	Li, H.	2014	Rabbit	36	M	Inlay (20)	BAM, rabbit (D)	Epithelial-differentiated rabbit adipose-derived stem cells	Yes
10	Wang, F.	2014	Rabbit	12	M	Inlay (10)	Denuded amnion, human (D)	Rabbit urothelial cells	Yes
11	Kajbafzadeh, A.M.	2014	Rabbit	12	M	Inlay (5)	Preputial acellular matrix, human (D)	-	Yes
12	Kawano, P.R.	2012	Rabbit	24	M	Inlay (10)	SIS, 1- and 4 –layer, porcine (D)	-	Yes
13	Guo, H.	2015	Rabbit	24	M	Inlay (20)	SIS, porcine (D)	Autologous keratinocytes and TIMP siRNA transfected fibroblasts	Yes
14	Kropp, B.P.	1998	Rabbit	8	M	Inlay(10)	SIS, porcine (D)	-	Yes

Table 1 (continued): Study characteristics of all 80 pre-clinical studies sorted on inclusion in meta-analysis, surgical procedure and biomaterial.

	Author	Year	Animal model	# of animals	Sex	Surgical Procedure (defect length in mm)	Biomaterial (category)	Type of added cells	Quality Assessment + Meta-analysis
15	Rotariu, P.	2002	Rabbit	7	M	Inlay (25)	SIS, porcine (D)	-	Yes
16	Villoldo, G.M.	2013	Rabbit	15	M	Inlay(10)	SIS, porcine (D)	-	Yes
17	Shokeir, A.	2003	Dog	21	M	Inlay (30)	UAM, dog (D)	-	Yes
18	Huang, J.W.	2015	Rabbit	30	M	Inlay (20)	Cellulose (N)	Rabbit Lingual keratinocytes	Yes
19	Xie, M.	2013	Dog	10	F	Inlay (50)	Silk fibroin (N)	Autologous oral keratinocytes and fibroblasts	Yes
20	Xie, M.	2013	Dog	9	F	Inlay (30)	Silk fibroin (N)	Dog urothelial cells	Yes
21	Sun, D.	2014	Rabbit	21	M	Inlay (5)	Subcutaneous implanted autologous minced muscle (N)	Human umbilical cord MSC's	Yes
22	Xu, Y.	2014	Rabbit	21	M+F	Inlay (5)	Subcutaneous implanted autologous muscle microsomes (N)	Human umbilical cord MSC's	Yes
23	Zhang, K.	2015	Rabbit	12	M	Inlay (20)	P(LA/CL) + type I collagen in combination with ICG-001 (Wnt-pathway inhibitor) (S)	Rabbit bladder urothelial cells	Yes
24	Wang, D.J.	2015	Rabbit	24	M	Inlay (5)	Polylactid acid (S)	Rabbit AdSC's	Yes
25	Kelami, A.	1971	Dog	10	M	Inlay (30)	PTFE (S) + lyophilized dura, human (D)	-	Yes
26	Chung, Y.G.	2014	Rabbit	8	M	Inlay (20)	Silk fibroin (N) + SIS (D)	-	Yes
27	Lv, X.	2016	Rabbit	18	M	Inlay (15)	Silk-Keratin-Gelatin-Calcium peroxide (N) + SIS, porcine (D)	-	Yes
28	Nuininga, J.E.	2003	Rabbit	18	M	Inlay (10)	SIS, 1 - and 4 - layer, porcine (D) + Type I collagen (N)	-	Yes
29	Zhang, Q.	2008	Rabbit	12	M	Full (10)	Acellular amnion, human (D)	Homologous endothelial progenitor cells	Yes
30	Parnigotto, P.P.	2000	Rabbit	12	M	Full (10)	Acellular aorta, rabbit (D)	-	Yes

Table 1 (continued): Study characteristics of all 80 pre-clinical studies sorted on inclusion in meta-analysis, surgical procedure and biomaterial.

	Author	Year	Animal model	# of animals	Sex	Surgical Procedure (defect length in mm)	Biomaterial (category)	Type of added cells	Quality Assessment + Meta-analysis
31	DeFilippo, R.E	2002	Rabbit	24	M	Full (10)	BAM (D)	Autologous bladder SMC's and urothelial cells	Yes
32	El-Tabey, N.	2012	Dog	14	F	Full (30)	BAM (D)	Autologous bladder SMC's and urothelial cells	Yes
33	Wang, J.H.	2013	Rabbit	18	M	Full (30)	BAM with polylactid acid –glycolic acid with VEGF (D)	-	Yes
34	DeFilippo, R.E.	2015	Rabbit	15	M	Full (30)	BAM, porcine (D)	Autologous bladder SMC's and urothelial cells	Yes
35	Dorin, R.P	2008	Rabbit	12	M	Full (5-30)	BAM, porcine (D)	-	Yes
36	Orabi, H.	2012	Dog	21	M	Full (60)	BAM, porcine (D)	Autologous bladder SMS's and urothelial cells	Yes
37	Fu, Q.	2007	Rabbit	18	M	Full (15)	BAM, rabbit (D)	Autologous foreskin epidermal cells	Yes
38	Fu, Q.	2008	Rabbit	18	M	Full (15)	BAM, rabbit (D)	Autologous foreskin epidermal cells	Yes
39	Gu, G.I.	2012	Rabbit	18	M	Full (15)	BAM, rabbit (D)	Autologous mesothelial cells	Yes
40	Li, C.L.	2013	Rabbit	30	M	Full (30)	BAM, rabbit (D)	Autologous bone-marrow derived MSC's and SMC's	Yes
41	Li, B.	2013	Rabbit	12	M	Full (15)	Frozen-thawed bladder mucosa, dog (D)	-	Yes
42	Kjaer, T.B.	1976	Dog	9	M	Full (30)	Lyophilized vein, dog (D)	-	Yes
43	Shokeir, A.	2004	Dog	14	M+F	Full (30)	UAM, dog (D)	-	Yes
44	Sievert, K.D.	2001	Rabbit	14	M	Full (8-11)	UAM, dog and rabbit (D)	-	Yes
45	Sievert, K.D.	2000	Rabbit	30	M	Full (8-11)	UAM, rabbit (D)	-	Yes
46	Lv, X.	2016	Dog	18	F	Full (20)	Bacterial cellulose + potato starch (N)	Dog lingual muscle cells	Yes

Table 1 (continued): Study characteristics of all 80 pre-clinical studies sorted on inclusion in meta-analysis, surgical procedure and biomaterial.

	Author	Year	Animal model	# of animals	Sex	Surgical Procedure (defect length in mm)	Biomaterial (category)	Type of added cells	Quality Assessment + Meta-analysis
47	Gu, G.I.	2010	Rabbit	9	M	Full (15)	De-novo created tissue in peritoneal cavity (N)	-	Yes
48	Jia, W.	2015	Dog	10	M	Full (50)	Type I collagen scaffold +/- 3VEGF (N)	-	Yes
49	A. Da Silva, L.F.	2014	Rabbit	16	M	Full (10)	Type I collagen, bovine (N)	Autologous bladder SMC's	Yes
50	Nuininga, J.E.	2010	Rabbit	32	M	Full (10)	Type I collagen, bovine (N)	-	Yes
51	Kanatani, I.	2007	Rabbit	28	M	Full (15)	Type I collagen, porcine + P(LA/CL) (N)	-	Yes
52	Micol, L.A.	2012	Rabbit	16	M	Full (10)	Type I collagen, rat tail (N)	Autologous bladder SMC's	Yes
53	Mikami, H.	2012	Dog	10	M	Full (20)	Type I collagen, rat tail (N)	Autologous oral epithelial and muscle cells	Yes
54	Italiano, G.	1997	Rabbit	14	M	Full (15)	Hyaluronan benzyl ester (S)	-	Yes
55	Italiano, G.	1998	Rabbit	4	M	Full (15)	Hyaluronan benzyl ester (S)	-	Yes
56	Fu, Q.	2014	Dog	18	M	Full (15)	PGA (S)	Oral mucosal epithelial cells and AdSC's	Yes
57	Hakky, S.I.	1977	Dog	15	M	Full (50)	Polyethylene terephthalate (S)	-	Yes
58	Hakky, S.I.	1977	Dog	9	M	Full (50)	Polyethylene terephthalate (S)	-	Yes
59	Olsen, L.	1992	Dog	6	M	Full (30-40)	Polyglactin fiber coated with polyhydroxybutyric acid (S)	-	Yes
60	Anwar, H.	1984	Dog	10	?	Full (25)	PTFE (S)	-	Yes
61	Dreikorn, K.	1979	Dog	12	M	Full (30-80)	PTFE (S)	-	Yes
63	El-Assmy, A.	2004	Rabbit	18	M	Inlay + Full (15)	SIS (D)	-	Yes
64	Wang, Y.Q.	2005	Rabbit	14	M	Inlay (10)	BAM, human (D)	-	No (CN)†
65	Beintker, M.	2007	Rat	20	M	Inlay (?)	SIS (D)	-	No*
66	Glybochko, P.V.	2014	Rabbit	Unknown	M	Full (?)	Acellular artery, human (D)	-	No (RU)†

Table 1 (continued): Study characteristics of all 80 pre-clinical studies sorted on inclusion in meta-analysis, surgical procedure and biomaterial.

	Author	Year	Animal model	# of animals	Sex	Surgical Procedure (defect length in mm)	Biomaterial (category)	Type of added cells	Quality Assessment + Meta-analysis
67	Peng, W.B.	2013	Rabbit	Unknown	M	Full (25)	BAM (D)	Rabbit hair follicle stem cells	No (CN)†
68	Hu, Y.F.	2008	Rabbit	30	M	Full (10-15)	UAM, rabbit (D)	-	No‡
69	Hu, Y.F.	2009	Rabbit	20	M	Full (10-15)	UAM, rabbit (D)	-	No (CN)†
70	Yang, S.X.	2004	Rabbit	30	M	Full (10-15)	UAM, rabbit (D)	-	No‡
71	Lebret, T.	1994	Rat	7	F	Full (?)	Type IV collagen, human (N)	-	No*
72	Fu, W.J.	2009	Rabbit	32	M	Full (10-15)	PLLA (S)	Autologous urothelial cells	No‡
73	Verit, A.	2003	Dog	2	M	Full (10)	PTFE (S)	-	No (TR)†
74	Huang, X.	2006	Rabbit	12	M	Inlay + Full (?)	SIS, porcine (D)	-	No (CN)†
75	Fu, Q.	2006	Rabbit	12	M	Unclear (10-30)	BAM, rabbit (D)	-	No (CN)†
76	Xu, L.S.	2007	Rabbit	48	M	Unclear (?)	UAM, porcine (D)	-	No‡
77	Han, P.	2009	Rabbit	24	M	Unclear (20)	UAM, rabbit (D)	Rabbit bladder SMC's	No (CN)†
78	Huang, H.J.	2007	Rabbit	48	M	Unclear (?)	UAM, rabbit (D)	Rabbit bone marrow derived MSC's	No (CN)†
79	Zhang, Y.	2011	Rabbit	Unknown	M	Unclear (?)	Silk fibroin (N)	Rabbit AdSC's	No (CN)†
80	Liu, C.	2008	Dog	12	M	Unclear (15-30)	Silk fibroin (N)	-	No (CN)†

† Excluded from meta-analysis due to language restrictions defined in section 2.5.

* Excluded from meta analysis because only two studies used rats (insufficient for statistical analysis).

‡ Excluded from meta analysis due to unclear experimental setup.

? = unclear, AdSC = adipose-derived stem cells, BAM = bladder acellular matrix, CN = Chinese, D = decellularized, MSC = mesenchymal stem cells, N = natural, P(LA/CL) = copoly(L-lactide/ε-caprolactone), PTFE = Polytetrafluoroethylene, RU = Russian, S = synthetic, SIS = small intestinal submucosa, SMC = Smooth muscle cell, TR = Turkish, UAM = urethral acellular matrix, VEGF = vascular endothelial growth factor.

Table 2: Study characteristics of all 23 clinical studies sorted on inclusion in meta-analysis, surgical procedure and biomaterial.

	Author	Year	Number of patients	Sex	Age range	Number of patients with prior surgery	Surgical procedure (defect length in mm)	Biomaterial (category)	Availability biomaterial	Type of added cells	Quality Assessment + Meta-analysis
1	Atala, A.	1999	4	M	4 – 20 y	4	Inlay (50-150)	BAM, human (D)	Exp	-	Yes
2	El-Kassaby, A.W.	2003	28	M	22 – 61 y	Unknown	Inlay (15-160)	BAM, human (D)	Exp	-	Yes
3	El-Kassaby, A.W.	2008	15	M	21 – 59 y	9	Inlay (20-180)	BAM, human (D)	Exp	-	Yes
4	Le Roux, P.J.	2005	9	M	15 – 56 y	5	Inlay (10-50)	SIS, porcine (D)	Com	-	Yes
5	Palminteri, E.	2006	20	M	20 – 74 y	16	Inlay (20-80)	SIS, porcine (D)	Com	-	Yes
6	Donkov, I.I.	2006	9	M	26 – 45 y	5	Inlay (40-60)	SIS, 4-layer, porcine (D)	Com	-	Yes
7	Fiala, R.	2007	50	M	45 – 73 y	Unknown	Inlay (40-140)	SIS, 4-layer, porcine (D)	Com	-	Yes
8	Orabi, H.	2013	12	M	1.5 – 15 y	3	Inlay (15-35)	SIS, 4-layer, porcine (D)	Com	-	Yes
9	Xu, Y.M.	2013	28	M	2 – 69 y	28	Inlay (35-70)	SIS, 4-layer, porcine (D)	Com	-	Yes
10	Hauser, S.	2006	5	M	61 – 80 y	5	Inlay (35-100)	SIS, 1- and 4 – layer, porcine (D)	Com	-	Yes
11	Osman, N.I.	2014	5	M	36 – 66 y	4	Inlay (?)	De-epidermised dermis, human (D)	Exp	Autologous buccal mucosa-derived keratinocytes and fibroblasts	Yes
12	Fossum, M.	2012	6	M	14 -44 m	Unknown	Inlay (?)	Acellular skin, human (D)	Exp	Autologous urothelial cells	Yes
13	Raya-Rivera, A.	2013	5	M	10 – 14 y	2	Full (40-60)	Polyglycolic acid and poly-(lactide-co-glycolide acid) (S)	Exp	Autologous bladder SMC's and urothelial cells	Yes

Table 2 (continued): Study characteristics of all 23 clinical studies sorted on inclusion in meta-analysis, surgical procedure and biomaterial.

	Author	Year	Number of patients	Sex	Age range	Number of patients with prior surgery	Surgical procedure (defect length in mm)	Biomaterial (category)	Availability biomaterial	Type of added cells	Quality Assessment + Meta-analysis
14	Bhargava, S.	2008	5	?	Unknown	5	Inlay (?)	De-epidermised dermis, human (D)	Exp	Autologous buccal mucosa-derived keratinocytes and fibroblasts	No [#]
15	Carpenter, C.P.	2012	1	M	68 y	1	Inlay (25)	Alloderm® (D)	Com	-	No‡
16	Kim, J.Y.	2005	1	M	48 y	0	Inlay (40)	Alloderm® (D)	Com	-	No‡
17	Mantovani, F.	2003	1	M	72 y	1	Inlay (?)	SIS, porcine (D)	Com	-	No‡
18	Lin, J.	2005	16	M	18 – 46 y	Unknown	Full (?)	Acellular skin, human (D)	Exp	-	No (CN)†
19	Villavicencio, H.	1989	22	?	28 – 80 y	Unknown	Inlay + full (?)	Lyophilized dura, human (D)	Exp	-	No (ES)†
20	Glybochko, P.	2015	1	M	64 y	Unknown	Unclear (?)	Acellular artery, human (D)	Exp	Autologous buccal mucosa-derived keratinocytes	No (RU)†
21	Yang, W.Z.	2011	8	M	4 – 23 y	0	Unclear (?)	Acellular skin human (D)	Exp	-	No*
22	Mantovani, F.	2002	5	M+F	70 – 79 y	Unknown	Unclear (?)	SIS, porcine (D)	Com	-	No (IT)†
23	Li, P.	2009	8	M	8 – 36 m	Unknown	Unclear (25-45)	Gelating sponge (N)	Exp	-	No (CN)†

[#] Excluded from meta-analysis because same patients were included in long-term follow-up study by Osman *et al.*, 2014 (#11).

[‡] Excluded from meta analysis due to case study.

† Excluded from meta-analysis due to language restrictions defined in section 2.5.

* Excluded from meta analysis due to unclear surgical procedure.

? = unclear, BAM = bladder acellular matrix, CN = Chinese, Com = Commercial availability, D = decellularized, ES = Spanish, Exp = Experimental availability, IT = Italian, MSC = mesenchymal stem cells, N = natural, RU = Russian, S = synthetic, SIS = small intestinal submucosa, SMC = Smooth muscle cell.

8. References

[1] Gallentine ML, Morey AF, Thompson IM, Jr. Hypospadias: a contemporary epidemiologic assessment. Urology. 2001;57:788-90.

[2] Pierik FH, Burdorf A, Nijman JM, et al. A high hypospadias rate in The Netherlands. Human reproduction (Oxford, England). 2002;17:1112-5.

[3] Mundy AR. Management of urethral strictures. Postgraduate medical journal. 2006;82:489-93.

[4] Brouwers MM, Feitz WF, Roelofs LA, et al. Risk factors for hypospadias. European journal of pediatrics. 2007;166:671-8.

[5] Santucci RA, Joyce GF, Wise M. Male urethral stricture disease. J Urol. 2007;177:1667-74.

[6] Orabi H, Bouhout S, Morissette A, et al. Tissue engineering of urinary bladder and urethra: advances from bench to patients. TheScientificWorldJournal. 2013;2013:154564.

[7] Lumen N, Oosterlinck W, Hoebeke P. Urethral reconstruction using buccal mucosa or penile skin grafts: systematic review and meta-analysis. Urol Int. 2012;89:387-94.

[8] Burger RA, Muller SC, el-Damanhoury H, et al. The buccal mucosal graft for urethral reconstruction: a preliminary report. J Urol. 1992;147:662-4.

[9] Barbagli G, Selli C, di Cello V, Mottola A. A one-stage dorsal free-graft urethroplasty for bulbar urethral strictures. Br J Urol. 1996;78:929-32.

[10] Dublin N, Stewart LH. Oral complications after buccal mucosal graft harvest for urethroplasty. BJU Int. 2004;94:867-9.

[11] Langer R, Vacanti JP. Tissue engineering. Science (New York, NY). 1993;260:920-6.

[12] Place ES, Evans ND, Stevens MM. Complexity in biomaterials for tissue engineering. Nature materials. 2009;8:457-70.

[13] Ma PX. Biomimetic materials for tissue engineering. Advanced Drug Delivery Reviews. 2008;60:184-98.

[14] Freed LE, Vunjak-Novakovic G, Biron RJ, et al. Biodegradable polymer scaffolds for tissue engineering. Bio/technology (Nature Publishing Company). 1994;12:689-93.

[15] Guo S, Dipietro LA. Factors affecting wound healing. Journal of dental research. 2010;89:219-29.

[16] Bianco P, Robey PG. Stem cells in tissue engineering. Nature. 2001;414:118-21.

[17] Sloff M, de Vries R, Geutjes P, et al. Tissue engineering in animal models for urinary diversion: a systematic review. PLoS One. 2014;9:e98734.

[18] Hooijmans CR, Tillema A, Leenaars M, Ritskes-Hoitinga M. Enhancing search efficiency by means of a search filter for finding all studies on animal experimentation in PubMed. Laboratory animals. 2010;44:170-5.

[19] de Vries RB, Hooijmans CR, Tillema A, Leenaars M, Ritskes-Hoitinga M. A search filter for increasing the retrieval of animal studies in Embase. Laboratory animals. 2011;45:268-70.

[20] Akaike H. Akaike's Information Criterion. In: Lovric M, editor. International Encyclopedia of Statistical Science. Berlin, Heidelberg: Springer Berlin Heidelberg; 2011. p. 25-.

[21] Zou GY. Assessment of risks by predicting counterfactuals. Statistics in medicine. 2009;28:3761-81.

[22] Barbagli G, Sansalone S, Djinovic R, Romano G, Lazzeri M. Current controversies in reconstructive surgery of the anterior urethra: a clinical overview. Int Braz J Urol. 2012;38:307-16; discussion 16.

[23] Mundy AR, Andrich DE. Urethral strictures. BJU Int. 2011;107:6-26.

[24] Subramaniam R, Spinoit AF, Hoebeke P. Hypospadias repair: an overview of the actual techniques. Seminars in plastic surgery. 2011;25:206-12.

[25] Singal AG, Higgins PD, Waljee AK. A primer on effectiveness and efficacy trials. Clinical and translational gastroenterology. 2014;5:e45.

[26] Faure A, Bouty A, Nyo YL, O'Brien M, Heloury Y. Two-stage graft urethroplasty for proximal and complicated hypospadias in children: A retrospective study. Journal of pediatric urology. 2016.

[27] Sloff M, Simaioforidis V, de Vries R, Oosterwijk E, Feitz W. Tissue engineering of the bladder--reality or myth? A systematic review. J Urol. 2014;192:1035-42.

[28] Dorin RP, Pohl HG, De Filippo RE, Yoo JJ, Atala A. Tubularized urethral replacement with unseeded matrices: what is the maximum distance for normal tissue regeneration? World J Urol. 2008:323-6.

[29] Qi N, Li WJ, Tian H. A systematic review of animal and clinical studies on the use of scaffolds for urethral repair. Journal of Huazhong University of Science and Technology Medical sciences = Hua zhong ke ji da xue xue bao Yi xue Ying De wen ban = Huazhong keji daxue xuebao Yixue Yingdewen ban. 2016;36:111-7.

[30] Xue JD, Gao J, Fu Q, Feng C, Xie H. Seeding cell approach for tissue-engineered urethral reconstruction in animal study: A systematic review and meta-analysis. Experimental biology and medicine (Maywood, NJ). 2016.

[31] Debels H, Hamdi M, Abberton K, Morrison W. Dermal matrices and bioengineered skin substitutes: a critical review of current options. Plastic and reconstructive surgery Global open. 2015;3:e284.

[32] de Vries RB, Buma P, Leenaars M, Ritskes-Hoitinga M, Gordijn B. Reducing the number of laboratory animals used in tissue engineering research by restricting the variety of animal models. Articular cartilage tissue engineering as a case study. Tissue engineering Part B, Reviews. 2012;18:427-35.

[33] de Vries RB, Wever KE, Avey MT, et al. The usefulness of systematic reviews of animal experiments for the design of preclinical and clinical studies. ILAR journal / National Research Council, Institute of Laboratory Animal Resources. 2014;55:427-37.

[34] Oerlemans AJ, Feitz WF, van Leeuwen E, Dekkers WJ. Regenerative urology clinical trials: an ethical assessment of road blocks and solutions. Tissue engineering Part B, Reviews. 2013;19:41-7.

[35] Hooijmans CR, Leenaars M, Ritskes-Hoitinga M. A gold standard publication checklist to improve the quality of animal studies, to fully integrate the Three Rs, and to make systematic reviews more feasible. Alternatives to laboratory animals : ATLA. 2010;38:167-82.

[36] Dixit R, Boelsterli UA. Healthy animals and animal models of human disease(s) in safety assessment of human pharmaceuticals, including therapeutic antibodies. Drug discovery today. 2007;12:336-42.

[37] Korevaar DA, Hooft L, ter Riet G. Systematic reviews and meta-analyses of preclinical studies: publication bias in laboratory animal experiments. Laboratory animals. 2011;45:225-30.

[38] Schneider CK, Salmikangas P, Jilma B, et al. Challenges with advanced therapy medicinal products and how to meet them. Nature reviews Drug discovery. 2010;9:195-201.

[39] EMA. Regulation (EC) No 1394/2007 of the European Parliament and of the Council on advanced therapy medicinal products and amending Directive 2001/83/EC and Regulation (EC) No 726/2004. 2007.

[40] Brevignon-Dodin L, Singh P. ATMP in practice: Towards a new industry landscape in tissue engineering.

[41] Culme-Seymour EJ, Mason K, Vallejo-Torres L, et al. Cost of Stem Cell-Based Tissue-Engineered Airway Transplants in the United Kingdom: Case Series. Tissue Eng Part A. 2016;22:208-13.

[42] Zhang Y, McNeill E, Tian H, et al. Urine derived cells are a potential source for urological tissue reconstruction. J Urol. 2008;180:2226-33.

Appendix 1

Search strategy to retrieve all relevant literature using PubMed. Individual search components for tissue engineering, urethra, pre-clinical- and clinical studies were designed using relevant MeSH terms and other relevant synonyms and related terms. Search components 1, 2 and 3A were combined to retrieve all relevant pre-clinical studies, while search components 1,2 and 3B were combined to retrieve all clinical studies.

Search component 1: Tissue Engineering

tissue engineering [MeSH] OR tissue culture techniques [MeSH] OR organ culture techniques [MeSH] OR organoids [MeSH] OR guided tissue regeneration [MeSH] OR regenerative medicine [MeSH] OR artificial organs [MeSH] OR tissue scaffolds [MeSH] OR biocompatible materials [MeSH] OR bioreactors [MeSH] OR (regenerative [tiab] AND (medicine [tiab] OR medicines [tiab])) OR ((decellularized [tiab] OR acellular [tiab] OR cell-free [tiab] bioartificial [tiab] OR bio-artificial [tiab] OR artificial [tiab] OR tissue [tiab] OR tissues [tiab] OR organ [tiab] OR organs [tiab] OR culture [tiab] OR cultures [tiab]) AND (autograft [tiab] OR autografts [tiab] OR graft [tiab] OR grafts [tiab] OR matrix [tiab] OR matrices [tiab] OR biomatrix [tiab] OR biomatrices [tiab] or biomaterial [tiab] OR biomaterials [tiab] OR scaffold [tiab] OR scaffolds [tiab] OR scaffolding [tiab] OR engineering [tiab] OR engineer [tiab] OR culture [tiab] OR cultures [tiab] OR regeneration [tiab] OR regenerated [tiab] OR regenerating [tiab] OR reconstruction [tiab] OR reconstructed [tiab] OR reconstructing [tiab])) OR tissue-engineered [tiab] OR tissue engineering [tiab] OR bio-engineering [tiab] OR bioengineering [tiab] OR bioengineered [tiab] OR bio-engineered [tiab] OR organoids [tiab] OR organoid [tiab] OR bioartificial [tiab] OR bio-artificial [tiab] OR artificial [tiab] OR scaffold [tiab] OR scaffolds [tiab] OR scaffolding [tiab] OR matrix [tiab] OR matrices [tiab] OR biomatrix [tiab] OR biomatrices [tiab] OR biomaterial [tiab] OR biomaterials [tiab] OR bioreactor [tiab] OR bioreactors [tiab]

Search component 2: Urethra

urethra [MeSH] OR urethral diseases [MeSH] OR urethra [tiab] OR urethral [tiab] OR urethras [tiab] OR urethrotomy [tiab] OR urethrotomies OR ureterotomy [tiab] OR ureterotomies [tiab] OR urethrotomia [tiab] OR urethroplasty OR urethroplasties [tiab] OR ((stricture [tiab] OR strictures [tiab]) AND (urology [tiab] OR urinary [tiab] OR urine [tiab] OR urological [tiab])) OR hypospadias [tiab] OR epispadias [tiab] OR urethritis [tiab] OR (meatus [tiab] AND (urology [tiab] OR urinary [tiab] OR urine [tiab] OR urological [tiab])) OR chordee [tiab] OR (perineal [tiab] AND (urology [tiab] OR urinary [tiab] OR urine [tiab] OR urological [tiab]))

Search component 3A: preclinical studies

("animal experimentation"[MeSH Terms] OR "models, animal"[MeSH Terms] OR "invertebrates"[MeSH Terms] OR "Animals"[Mesh:noexp] OR "animal population groups"[MeSH Terms] OR "chordata"[MeSH Terms:noexp] OR "chordata, nonvertebrate"[MeSH Terms] OR "vertebrates"[MeSH Terms:noexp] OR "amphibians"[MeSH Terms] OR "birds"[MeSH Terms] OR "fishes"[MeSH Terms] OR "reptiles"[MeSH Terms] OR "mammals"[MeSH Terms:noexp] OR "primates"[MeSH Terms:noexp] OR "artiodactyla"[MeSH Terms] OR "carnivora"[MeSH Terms] OR "cetacea"[MeSH Terms] OR "chiroptera"[MeSH Terms] OR "elephants"[MeSH Terms] OR "hyraxes"[MeSH Terms] OR "insectivora"[MeSH Terms] OR "lagomorpha"[MeSH Terms] OR

"marsupialia"[MeSH Terms] OR "monotremata"[MeSH Terms] OR "perissodactyla"[MeSH Terms] OR "rodentia"[MeSH Terms] OR "scandentia"[MeSH Terms] OR "sirenia"[MeSH Terms] OR "xenarthra"[MeSH Terms] OR "haplorhini"[MeSH Terms:noexp] OR "strepsirhini"[MeSH Terms] OR "platyrrhini"[MeSH Terms] OR "tarsii"[MeSH Terms] OR "catarrhini"[MeSH Terms:noexp] OR "cercopithecidae"[MeSH Terms] OR "hylobatidae"[MeSH Terms] OR "hominidae"[MeSH Terms:noexp] OR "gorilla gorilla"[MeSH Terms] OR "pan paniscus"[MeSH Terms] OR "pan troglodytes"[MeSH Terms] OR "pongo pygmaeus"[MeSH Terms]) OR ((animals[tiab] OR animal[tiab] OR mice[Tiab] OR mus[Tiab] OR mouse[Tiab] OR murine[Tiab] OR woodmouse[tiab] OR rats[Tiab] OR rat[Tiab] OR murinae[Tiab] OR muridae[Tiab] OR cottonrat[tiab] OR cottonrats[tiab] OR hamster[tiab] OR hamsters[tiab] OR cricetinae[tiab] OR rodentia[Tiab] OR rodent[Tiab] OR rodents[Tiab] OR pigs[Tiab] OR pig[Tiab] OR swine[tiab] OR swines[tiab] OR piglets[tiab] OR piglet[tiab] OR boar[tiab] OR boars[tiab] OR "sus scrofa"[tiab] OR ferrets[tiab] OR ferret[tiab] OR polecat[tiab] OR polecats[tiab] OR "mustela putorius"[tiab] OR "guinea pigs"[Tiab] OR "guinea pig"[Tiab] OR cavia[Tiab] OR callithrix[Tiab] OR marmoset[Tiab] OR marmosets[Tiab] OR cebuella[Tiab] OR hapale[Tiab] OR octodon[Tiab] OR chinchilla[Tiab] OR chinchillas[Tiab] OR gerbillinae[Tiab] OR gerbil[Tiab] OR gerbils[Tiab] OR jird[Tiab] OR jirds[Tiab] OR merione[Tiab] OR meriones[Tiab] OR rabbits[Tiab] OR rabbit[Tiab] OR hares[Tiab] OR hare[Tiab] OR diptera[Tiab] OR flies[Tiab] OR fly[Tiab] OR dipteral[Tiab] OR drosophila[Tiab] OR drosophilidae[Tiab] OR cats[Tiab] OR cat[Tiab] OR carus[Tiab] OR felis[Tiab] OR nematoda[Tiab] OR nematode[Tiab] OR nematodes[Tiab] OR sipunculida[Tiab] OR dogs[Tiab] OR dog[Tiab] OR canine[Tiab] OR canines[Tiab] OR canis[Tiab] OR sheep[Tiab] OR sheeps[Tiab] OR mouflon[Tiab] OR mouflons[Tiab] OR ovis[Tiab] OR goats[Tiab] OR goat[Tiab] OR capra[Tiab] OR capras[Tiab] OR rupicapra[Tiab] OR rupicapras[Tiab] OR chamois[Tiab] OR haplorhini[Tiab] OR monkey[Tiab] OR monkeys[Tiab] OR anthropoidea[Tiab] OR anthropoids[Tiab] OR saguinus[Tiab] OR tamarin[Tiab] OR tamarins[Tiab] OR leontopithecus[Tiab] OR hominidae[Tiab] OR ape[Tiab] OR apes[Tiab] OR "pan paniscus"[Tiab] OR bonobo[Tiab] OR bonobos[Tiab] OR "pan troglodytes"[Tiab] OR gibbon[Tiab] OR gibbons[Tiab] OR siamang[Tiab] OR siamangs[Tiab] OR nomascus[Tiab] OR symphalangus[Tiab] OR chimpanzee[Tiab] OR chimpanzees[Tiab] OR prosimian[Tiab] OR prosimians[Tiab] OR "bush baby"[Tiab] OR bush babies[Tiab] OR galagos[Tiab] OR galago[Tiab] OR pongidae[Tiab] OR gorilla[Tiab] OR gorillas[Tiab] OR "pongo pygmaeus"[Tiab] OR orangutan[Tiab] OR orangutans[Tiab] OR lemur[Tiab] OR lemurs[Tiab] OR lemuridae[Tiab] OR horse[Tiab] OR horses[Tiab] OR equus[Tiab] OR cow[Tiab] OR calf[Tiab] OR bull[Tiab] OR chicken[Tiab] OR chickens[Tiab] OR gallus[Tiab] OR quail[Tiab] OR bird[Tiab] OR birds[Tiab] OR quails[Tiab] OR poultry[Tiab] OR poultries[Tiab] OR fowl[Tiab] OR fowls[Tiab] OR reptile[Tiab] OR reptilia[Tiab] OR reptiles[Tiab] OR snakes[Tiab] OR snake[Tiab] OR lizard[Tiab] OR lizards[Tiab] OR alligator[Tiab] OR alligators[Tiab] OR crocodile[Tiab] OR crocodiles[Tiab] OR turtle[Tiab] OR turtles[Tiab] OR amphibian[Tiab] OR amphibians[Tiab] OR amphibia[Tiab] OR frog[Tiab] OR frogs[Tiab] OR bombina[Tiab] OR salientia[Tiab] OR toad[Tiab] OR toads[Tiab] OR "epidalea calamita"[Tiab] OR salamander[Tiab] OR salamanders[Tiab] OR eel[Tiab] OR eels[Tiab] OR fish[Tiab] OR fishes[Tiab] OR pisces[Tiab] OR catfish[Tiab] OR catfishes[Tiab] OR siluriformes[Tiab] OR arius[Tiab] OR heteropneustes[Tiab] OR sheatfish[Tiab] OR perch[Tiab] OR perches[Tiab] OR percidae[Tiab] OR perca[Tiab] OR trout[Tiab] OR trouts[Tiab] OR char[Tiab] OR chars[Tiab] OR salvelinus[Tiab] OR minnow[Tiab] OR cyprinidae[Tiab] OR carps[Tiab] OR carp[Tiab] OR zebrafish[Tiab] OR zebrafishes[Tiab] OR goldfish[Tiab] OR goldfishes[Tiab] OR guppy[Tiab] OR guppies[Tiab] OR chub[Tiab] OR chubs[Tiab] OR tinca[Tiab] OR barbels[Tiab] OR barbus[Tiab] OR pimephales[Tiab] OR promelas[Tiab] OR "poecilia reticulata"[Tiab] OR mullet[Tiab] OR mullets[Tiab] OR eel[Tiab] OR eels[Tiab] OR seahorse[Tiab] OR seahorses[Tiab] OR mugil curema[Tiab] OR atlantic cod[Tiab] OR shark[Tiab] OR sharks[Tiab] OR catshark[Tiab] OR anguilla[Tiab] OR salmonid[Tiab] OR salmonids[Tiab] OR whitefish[Tiab] OR whitefishes[Tiab] OR salmon[Tiab] OR salmons[Tiab] OR sole[Tiab] OR solea[Tiab] OR lamprey[Tiab] OR lampreys[Tiab] OR pumpkinseed[Tiab] OR sunfish[Tiab] OR sunfishes[Tiab] OR tilapia[Tiab] OR

tilapias[Tiab] OR turbot[Tiab] OR turbots[Tiab] OR flatfish[Tiab] OR flatfishes[Tiab] OR sciuridae[Tiab] OR squirrel[Tiab] OR squirrels[Tiab] OR chipmunk[Tiab] OR chipmunks[Tiab] OR suslik[Tiab] OR susliks[Tiab] OR vole[Tiab] OR voles[Tiab] OR lemming[Tiab] OR lemmings[Tiab] OR muskrat[Tiab] OR muskrats[Tiab] OR lemmus[Tiab] OR otter[Tiab] OR otters[Tiab] OR marten[Tiab] OR martens[Tiab] OR martes[Tiab] OR weasel[Tiab] OR badger[Tiab] OR badgers[Tiab] OR ermine[Tiab] OR mink[Tiab] OR minks[Tiab] OR sable[Tiab] OR sables[Tiab] OR gulo[Tiab] OR gulos[Tiab] OR wolverine[Tiab] OR wolverines[Tiab] OR mustela[Tiab] OR llama[Tiab] OR llamas[Tiab] OR alpaca[Tiab] OR alpacas[Tiab] OR camelid[Tiab] OR camelids[Tiab] OR guanaco[Tiab] OR guanacos[Tiab] OR chiroptera[Tiab] OR chiropteras[Tiab] OR bat[Tiab] OR bats[Tiab] OR fox[Tiab] OR foxes[Tiab] OR iguana[Tiab] OR iguanas[Tiab] OR xenopus laevis[Tiab] OR parakeet[Tiab] OR parakeets[Tiab] OR parrot[Tiab] OR parrots[Tiab] OR donkey[Tiab] OR donkeys[Tiab] OR mule[Tiab] OR mules[Tiab] OR zebra[Tiab] OR zebras[Tiab] OR shrew[Tiab] OR shrews[Tiab] OR bison[Tiab] OR bisons[Tiab] OR buffalo[Tiab] OR buffaloes[Tiab] OR deer[Tiab] OR deers[Tiab] OR bear[Tiab] OR bears[Tiab] OR panda[Tiab] OR pandas[Tiab] OR “wild hog”[Tiab] OR “wild boar”[Tiab] OR fitchew[Tiab] OR fitch[Tiab] OR beaver[Tiab] OR beavers[Tiab] OR jerboa[Tiab] OR jerboas[Tiab] OR capybara[Tiab] OR capybaras[Tiab]) NOT medline[sb])

Search component 3B: clinical studies

(((clinical[Title/Abstract] AND trial[Title/Abstract]) OR clinical trials[MeSH Terms] OR clinical trial[Publication Type] OR random*[Title/Abstract] OR random allocation[MeSH Terms] OR therapeutic use[MeSH Subheading])) OR ((Human[tiab] OR humans[tiab] OR patient[tiab] OR patients[tiab]) AND (study[tiab] OR studies[tiab] OR research[tiab] OR investigat*[tiab] OR clinic*[tiab] OR therapy[tiab] OR treatment[tiab] OR surgery[tiab] OR surgical[tiab]))

Appendix 2

Search strategy to retrieve all relevant literature using Embase. Individual search components for tissue engineering, urethra, pre-clinical- and clinical studies were designed using relevant EMTree-terms and relevant synonyms and related terms. Search components 1, 2 and 3A were combined to retrieve all relevant pre-clinical studies, while search components 1,2 and 3B were combined to retrieve all clinical studies.

Search component 1: Tissue Engineering

Exp tissue engineering/ OR Exp tissue culture/ OR Exp organ culture/ OR Exp tissue regeneration/ OR Exp regenerative medicine/ OR Exp artificial organ/ OR Exp tissue scaffold/ OR Exp biomaterial/ OR Exp bioreactor/ OR (regenerative AND (medicine OR medicines)).ti,ab. OR ((decellularized OR acellular OR cell-free bioartificial OR bio-artificial OR artificial OR tissue OR tissues OR organ OR organs OR culture OR cultures) AND (autograft OR autografts OR graft OR grafts OR matrix OR matrices OR biomatrix OR biomatrices OR biomaterial OR biomaterials OR scaffold OR scaffolds OR scaffolding OR engineering OR engineer OR culture OR cultures OR regeneration OR regenerated OR regenerating OR reconstruction OR reconstructed OR reconstructing)).ti,ab. OR (tissue-engineered OR tissue engineering OR bio-engineering OR bioengineering OR bioengineered OR bio-engineered OR organoids OR organoid OR bioartificial OR

bio-artificial OR artificial OR scaffold OR scaffolds OR scaffolding OR matrix OR matrices OR biomatrix OR biomatrices OR biomaterial OR biomaterials OR bioreactor OR bioreactors).ti,ab.

Search component 2: Urethra

Exp urethra/ OR Exp urethra disease/ OR Exp urethra surgery/ OR urethra.ti,ab. OR urethral.ti,ab. OR urethras.ti,ab. OR urethrotomy.ti,ab. OR urethrotomies.ti,ab. OR ureterotomy.ti,ab. OR ureterotomies.ti,ab. OR urethrotomia.ti,ab. OR urethroplasty.ti,ab. OR urethroplasties.ti,ab. OR ((stricture OR strictures) AND (urology OR urinary OR urine OR urological)).ti,ab. OR hypospadias.ti,ab. OR epispadias.ti,ab. OR urethritis.ti,ab. OR (meatus AND (urology OR urinary OR urine OR urological)).ti,ab. OR chordee.ti,ab. OR (perineal AND (urology OR urinary OR urine OR urological)).ti,ab.

Search component 3A: preclinical studies

exp animal experiment/ or exp animal model/ or exp experimental animal/ or exp transgenic animal/ or exp male animal/ or exp female animal/ or exp juvenile animal/ OR animal/ OR chordata/ OR vertebrate/ OR tetrapod/ OR exp fish/ OR amniote/ OR exp amphibia/ OR mammal/ OR exp reptile/ OR exp sauropsid/ OR therian/OR exp monotremate/ OR placental mammals/ OR exp marsupial/ OR Euarchontoglires/ OR exp Afrotheria/ OR exp Boreoeutheria/ OR exp Laurasiatheria/ OR exp Xenarthra/ OR primate/ OR exp Dermoptera/ OR exp Glires/ OR exp Scandentia/ OR Haplorhini/ OR exp prosimian/ OR simian/ OR exp tarsiiform/ OR Catarrhini/ OR exp Platyrrhini/ OR ape/ OR exp Cercopithecidae/ OR hominid/ OR exp hylobatidae/ OR exp chimpanzee/ OR exp gorilla/ OR exp orang utan/ OR (animal OR animals OR pisces OR fish OR fishes OR catfish OR catfishes OR sheatfish OR silurus OR arius OR heteropneustes OR clarias OR gariepinus OR fathead minnow OR fathead minnows OR pimephales OR promelas OR cichlidae OR trout OR trouts OR char OR chars OR salvelinus OR salmo OR oncorhynchus OR guppy OR guppies OR millionfish OR poecilia OR goldfish OR goldfishes OR carassius OR auratus OR mullet OR mullets OR mugil OR curema OR shark OR sharks OR cod OR cods OR gadus OR morhua OR carp OR carps OR cyprinus OR carpio OR killifish OR eel OR eels OR anguilla OR zander OR sander OR lucioperca OR stizostedion OR turbot OR turbots OR psetta OR flatfish OR flatfishes OR plaice OR pleuronectes OR platessa OR tilapia OR tilapias OR oreochromis OR sarotherodon OR common sole OR dover sole OR solea OR zebrafish OR zebrafishes OR danio OR rerio OR seabass OR dicentrarchus OR labrax OR morone OR lamprey OR lampreys OR petromyzon OR pumpkinseed OR pumpkinseeds OR lepomis OR gibbosus OR herring OR clupea OR harengus OR amphibia OR amphibian OR amphibians OR anura OR salientia OR frog OR frogs OR rana OR toad OR toads OR bufo OR xenopus OR laevis OR bombina OR epidalea OR calamita OR salamander OR salamanders OR newt OR newts OR triturus OR reptilia OR reptile OR reptiles OR bearded dragon OR pogona OR vitticeps OR iguana OR iguanas OR lizard OR lizards OR anguis fragilis OR turtle OR turtles OR snakes OR snake OR aves OR bird OR birds OR quail OR quails OR coturnix OR bobwhite OR colinus OR virginianus OR poultry OR poultries OR fowl OR fowls OR chicken OR chickens OR gallus OR zebra finch OR taeniopygia OR guttata OR canary OR canaries OR serinus OR canaria OR parakeet OR parakeets OR grasskeet OR parrot OR parrots OR psittacine OR psittacines OR shelduck OR tadorna OR goose OR geese OR branta OR leucopsis OR woodlark OR lullula OR flycatcher OR ficedula OR hypoleuca OR dove OR doves OR geopelia OR cuneata OR duck OR ducks OR greylag OR graylag OR anser OR harrier OR circus pygargus OR red knot OR great knot OR calidris OR canutus OR godwit OR limosa OR lapponica OR meleagris OR gallopavo OR jackdaw OR corvus OR monedula OR ruff OR philomachus OR pugnax OR lapwing OR peewit OR plover OR vanellus OR swan OR cygnus OR columbianus OR bewickii OR gull OR chroicocephalus OR ridibundus OR albifrons OR great tit OR parus OR aythya OR fuligula OR streptopelia OR risoria OR spoonbill OR platalea OR leucorodia OR blackbird OR turdus OR merula OR blue tit OR cyanistes OR pigeon OR pigeons OR

columba OR pintail OR anas OR starling OR sturnus OR owl OR athene noctua OR pochard OR ferina OR cockatiel OR nymphicus OR hollandicus OR skylark OR alauda OR tern OR sterna OR teal OR crecca OR oystercatcher OR haematopus OR ostralegus OR shrew OR shrews OR sorex OR araneus OR crocidura OR russula OR european mole OR talpa OR chiroptera OR bat OR bats OR eptesicus OR serotinus OR myotis OR dasycneme OR daubentonii OR pipistrelle OR pipistrellus OR cat OR cats OR felis OR catus OR feline OR dog OR dogs OR canis OR canine OR canines OR otter OR otters OR lutra OR badger OR badgers OR meles OR fitchew OR fitch OR foumart or foulmart OR ferrets OR ferret OR polecat OR polecats OR mustela OR putorius OR weasel OR weasels OR fox OR foxes OR vulpes OR common seal OR phoca OR vitulina OR grey seal OR halichoerus OR horse OR horses OR equus OR equine OR equidae OR donkey OR donkeys OR mule OR mules OR pig OR pigs OR swine OR swines OR hog OR hogs OR boar OR boars OR porcine OR piglet OR piglets OR sus OR scrofa OR llama OR llamas OR lama OR glama OR deer OR deers OR cervus OR elaphus OR cow OR cows OR bos taurus OR bos indicus OR bovine OR bull OR bulls OR cattle OR bison OR bisons OR sheep OR sheeps OR ovis aries OR ovine OR lamb OR lambs OR mouflon OR mouflons OR goat OR goats OR capra OR caprine OR chamois OR rupicapra OR leporidae OR lagomorpha OR lagomorph OR rabbit OR rabbits OR oryctolagus OR cuniculus OR laprine OR hares OR lepus OR rodentia OR rodent OR rodents OR murinae OR mouse OR mice OR mus OR musculus OR murine OR woodmouse OR apodemus OR rat OR rats OR rattus OR norvegicus OR guinea pig OR guinea pigs OR cavia OR porcellus OR hamster OR hamsters OR mesocricetus OR cricetulus OR cricetus OR gerbil OR gerbils OR jird OR jirds OR meriones OR unguiculatus OR jerboa OR jerboas OR jaculus OR chinchilla OR chinchillas OR beaver OR beavers OR castor fiber OR castor canadensis OR sciuridae OR squirrel OR squirrels OR sciurus OR chipmunk OR chipmunks OR marmot OR marmots OR marmota OR suslik OR susliks OR spermophilus OR cynomys OR cottonrat OR cottonrats OR sigmodon OR vole OR voles OR microtus OR myodes OR glareolus OR primate OR primates OR prosimian OR prosimians OR lemur OR lemurs OR lemuridae OR loris OR bush baby OR bush babies OR bushbaby OR bushbabies OR galago OR galagos OR anthropoidea OR anthropoids OR simian OR simians OR monkey OR monkeys OR marmoset OR marmosets OR callithrix OR cebuella OR tamarin OR tamarins OR saguinus OR leontopithecus OR squirrel monkey OR squirrel monkeys OR saimiri OR night monkey OR night monkeys OR owl monkey OR owl monkeys OR douroucoulis OR aotus OR spider monkey OR spider monkeys OR ateles OR baboon OR baboons OR papio OR rhesus monkey OR macaque OR macaca OR mulatta OR cynomolgus OR fascicularis OR green monkey OR green monkeys OR chlorocebus OR vervet OR vervets OR pygerythrus OR hominoidea OR ape OR apes OR hylobatidae OR gibbon OR gibbons OR siamang OR siamangs OR nomascus OR symphalangus OR hominidae OR orangutan OR orangutans OR pongo OR chimpanzee OR chimpanzees OR pan troglodytes OR bonobo OR bonobos OR pan paniscus OR gorilla OR gorillas OR troglodytes).ti,ab.

Search component 3B: clinical studies

Exp clinical trial/ OR (clinical AND (trial OR trials)).ti,ab. OR Exp randomization/ OR Exp therapy/ OR Exp treatment outcome/ OR Exp controlled study/ OR exp health care quality/ OR random:.tw. or clinical trial:.mp.

Appendix 3

Reference list on alphabetical order of preclinical studies included in Table 1.

Anwar H, Dave B, Seebode JJ. Replacement of partially resected canine urethra by polytetrafluoroethylene. Urology. 1984;24:583-6.

Arenas da Silva LF, Micol L, Tiemessen D, van Kuppevelt TH, Frey P, Oosterwijk E, et al. Is there a need for smooth muscle cell transplantation in urethral reconstruction? Tissue Eng Part A. 2013.

Ayyildiz A, Celebi B, Akgul KT, Nuhoglu B, Caydere M, Germiyanoglu C. A comparison of free skin graft, fascia lata, alloderm, bovine pericardium and primary repair in urethrocutaneous fistulas without diversion: an experimental study. Pediatr Surg Int. 2006:809-14.

Beintker M, Vogt B, Schubert J, Schubert H, Kosmehl H. Urethroplasty with tissue matrix. Microsurgical animal experiments with small intestinal submucosa. [German]. Urologe - Ausgabe A. 2007:1236-7.

Chen F, Yoo JJ, Atala A. Acellular collagen matrix as a possible "off the shelf" biomaterial for urethral repair. Urology. 1999:407-10.

Chun SY, Kim BS, Kwon SY, Park SI, Song PH, Yoo ES, et al. Urethroplasty using autologous urethral tissue-embedded acellular porcine bladder submucosa matrix grafts for the management of long-segment urethral stricture in a rabbit model. Journal of Korean medical science. 2015;30:301-7.

Chung YG, Tu D, Franck D, Gil ES, Algarrahi K, Adam RM, et al. Acellular bi-layer silk fibroin scaffolds support tissue regeneration in a rabbit model of onlay urethroplasty. PLoS ONE. 2014;9.

De Filippo RE, Yoo JJ, Atala A. Urethral replacement using cell seeded tubularized collagen matrices. J Urol. 2002:1789-92; discussion 92-3.

De Filippo RE, Kornitzer BS, Yoo JJ, Atala A. Penile urethra replacement with autologous cell-seeded tubularized collagen matrices. Journal of Tissue Engineering and Regenerative Medicine. 2015;9:257-64.

Dorin RP, Pohl HG, De Filippo RE, Yoo JJ, Atala A. Tubularized urethral replacement with unseeded matrices: what is the maximum distance for normal tissue regeneration? World J Urol. 2008:323-6.

Dreikorn K, Loebelenz J, Horsch R, Roehl L. Alloplastic replacement of the partially resected canine urethra by expanded polytetrafluoroethylene grafts. Urol Res. 1979:19-21.

El-Assmy A, El-Hamid MA, Hafez AT. Urethral replacement: a comparison between small intestinal submucosa grafts and spontaneous regeneration. BJU Int. 2004:1132-5.

El-Tabey N, Shokeir A, Barakat N, El-Refaie H, El-Hamid MA, Gabr M. Cell-seeded tubular acellular matrix for replacing a long circumferential urethral defect in a canine model: Is it clinically applicable? Arab Journal of Urology. 2012:192-8.

Feng C, Xu YM, Fu Q, Zhu WD, Cui L. Reconstruction of three dimensional neo-urethra using lingual keratinocytes & corporal smooth muscle cells seeded acellular corporal spongiosum. Journal of Urology. 2011:e7.

Fu Q, Den C, Yin D, Cui L, Liu W, Cao Y. The experimental study of applying acellular bladder submucosa as a biom aterial for urethral defect repair. [Chinese]. Chinese Journal of Andrology. 2006:18-22.

Fu Q, Deng CL, Liu W, Cao YL. Urethral replacement using epidermal cell-seeded tubular acellular bladder collagen matrix. BJU Int. 2007:1162-5.

Fu Q, Deng CL, Song XF, Xu YM. Long-term study of male rabbit urethral mucosa reconstruction using epidermal cell. Asian J Androl. 2008:719-22.

Fu Q, Deng CL, Zhao RY, Wang Y, Cao Y. The effect of mechanical extension stimulation combined with epithelial cell sorting on outcomes of implanted tissue-engineered muscular urethras. Biomaterials. 2014:105-12.

Fu WJ, Zhang X, Zhang BH, Zhang P, Hong BF, Gao JP, et al. Biodegradable urethral stents seeded with autologous urethral epithelial cells in the treatment of post-traumatic urethral stricture: a feasibility study in a rabbit model. BJU Int. 2009:263-8.

Glybochko PV, Aliaev Iu G, Nikolenko VN, Shekhter AB, Vinarov AZ, Istranov LP, et al. [Experimental validation of the developing a matrix based on decellularized vascular wall for subsequent substitution urethroplasty]. Urologiia (Moscow, Russia : 1999). 2014:41-6.

Gu GL, Zhu YJ, Xia SJ, Zhang J, Jiang JT, Hong Y, et al. Peritoneal cavity as bioreactor to grow autologous tubular urethral grafts in a rabbit model. World J Urol. 2010:227-32.

Gu GL, Xia SJ, Zhang J, Liu GH, Yan L, Xu ZH, et al. Tubularized urethral replacement using tissue-engineered peritoneum-like tissue in a rabbit model. Urol Int. 2012:358-64.

Guo H, Sa Y, Huang J, Wang Z, Wang L, Xie M, et al. Urethral Reconstruction with Small Intestinal Submucosa Seeded with Oral Keratinocytes and TIMP-1 siRNA Transfected Fibroblasts in a Rabbit Model. Urologia Internationalis. 2016;96:223-30.

Hakky SI. The use of fine double siliconised dacron in urethral replacement. Br J Urol. 1977:167-71.

Hakky SI. Br J Urol. 1977:729-32.

Han P, Song C, Yang YR, Wei Q, Li H, Wang KJ. Urethral acellular matrix graft for repairing urethral defect in rabbits. [Chinese]. Nan Fang Yi Ke Da Xue Xue Bao. 2009:124-7, 32.

Hu YF, Yang SX, Wang LL, Jin HM. Curative effect and histocompatibility evaluation of reconstruction of traumatic defect of rabbit urethra using extracellular matrix. Chin J Traumatol. 2008:274-8.

Hu YF, Yang SX, Wang LL, Jin HM, Zhan BY. Reconstruction of rabbit urethra using urethral extracellular matrix. [Chinese]. Zhonghua Zheng Xing Wai Ke Za Zhi. 2009:54-7.

Huang HJ, Zhang JM, Chu HH. Feasibility of constructing tissue-engineered corpus cavernous urethra with rabbit bone marrow mesenchymal stem cells. [Chinese]. Journal of Clinical Rehabilitative Tissue Engineering Research. 2007:2665-8.

Huang JW, Xie MK, Zhang Y, Wei GJ, Li X, Li HB, et al. Reconstruction of penile urethra with the 3-dimensional porous bladder acellular matrix in a rabbit model. Urology. 2014;84:1499-505.

Huang JW, Lv XG, Li Z, Song LJ, Feng C, Xie MK, et al. Urethral reconstruction with a 3D porous bacterial cellulose scaffold seeded with lingual keratinocytes in a rabbit model. Biomedical materials (Bristol, England). 2015;10:055005.

Huang X, Luo J, Liao Y, Qu Y, Yang Z. Study on small intestinal submucosa as repair materials in urethral reconstruction. [Chinese]. Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi. 2006:206-9.

Italiano G, Abatangelo G, Jr., Calabro A, Abatangelo G, Sr., Zanoni R, Regan M, et al. Reconstructive surgery of the urethra: a pilot study in the rabbit on the use of hyaluronan benzyl ester (Hyaff-11) biodegradable grafts. Urol Res. 1997:137-42.

Italiano G, Abatangelo G, Jr., Calabro A, Zanoni R, Abatangelo G, Sr., Passerini-Glazel G. Guiding spontaneous tissue regeneration for urethral reconstruction: long-term studies in the rabbit. Urol Res. 1998:281-4.

Jia W, Tang H, Wu J, Hou X, Chen B, Chen W, et al. Urethral tissue regeneration using collagen scaffold modified with collagen binding VEGF in a beagle model. Biomaterials. 2015;69:45-55.

Kajbafzadeh AM, Sabetkish S, Tourchi A, Amirizadeh N, Afshar K, Abolghasemi H, et al. The application of tissue-engineered preputial matrix and fibrin sealant for urethral reconstruction in rabbit model. International Urology and Nephrology. 2014;46:1573-80.

Kanatani I, Kanematsu A, Inatsugu Y, Imamura M, Negoro H, Ito N, et al. Fabrication of an optimal urethral graft using collagen-sponge tubes reinforced with copoly(L-lactide/-caprolactone) fabric. Tissue Eng. 2007:2933-40.

Kawano PR, Fugita OE, Yamamoto HA, Quitzan JG, Padovani C, Amaro JL. Comparative study between porcine small intestinal submucosa and buccal mucosa in a partial urethra substitution in rabbits. J Endourol. 2012:427-32.

Kelami A, Korb G, Ludtke-Handjery A, Rolle J, Schnell J, Lehnhardt FH. Alloplastic replacement of the partially resected urethra on dogs. Invest Urol. 1971:55-8.

Kjaer TB, Nilsson T, Madsen PO. Total replacement of part of the canine urethra with lyophilized vein homografts. Invest Urol. 1976:159-61.

Kropp BP, Ludlow JK, Spicer D, Rippy MK, Badylak SF, Adams MC, et al. Rabbit urethral regeneration using small intestinal submucosa onlay grafts. Urology. 1998:138-42.

Lebret T, Gobet F, Dallaserra M, Mitrofanoff P. Urethroplasty of the free colonic mucosa in rats. Preliminary study of the use of appendicular mucosa. [French]. Chirurgie. 1994:479-85.

Li B, Lu Y, Liu C, Zheng S, Li H, Pu J, et al. Urethral reconstruction using allogenic frozen-thawed bladder mucosa: an experimental study. Urol Int. 2013:422-9.

Li C, Xu YM, Song LJ, Fu Q, Cui L, Yin S. Urethral Reconstruction Using Oral Keratinocyte Seeded Bladder Acellular Matrix Grafts. Journal of Urology. 2008:1538-42.

Li C, Xu YM, Liu ZS, Li HB. Urethral reconstruction with tissue engineering and RNA interference techniques in rabbits. Urology. 2013:1075-80.

Li CL, Liao WB, Yang SX, Song C, Li YW, Xiong YH, et al. Urethral reconstruction using bone marrow mesenchymal stem cell- and smooth muscle cell-seeded bladder acellular matrix. Transplant Proc. 2013:3402-7.

Li H, Xu Y, Xie H, Li C, Song L, Feng C, et al. Epithelial-differentiated adipose-derived stem cells seeded bladder acellular matrix grafts for urethral reconstruction: an animal model. Tissue Eng Part A. 2014:774-84.

Liu CX, Chen WH, Zheng SB, Li HL. Different lengths of dog urethral defects repaired with Silk fibroin film. [Chinese]. Journal of Clinical Rehabilitative Tissue Engineering Research. 2008:3613-6.

Lv X, Li Z, Chen S, Xie M, Huang J, Peng X, et al. Structural and functional evaluation of oxygenating keratin/silk fibroin scaffold and initial assessment of their potential for urethral tissue engineering. Biomaterials. 2016;84:99-110.

Lv X, Yang J, Feng C, Li Z, Chen S, Xie M, et al. Bacterial Cellulose-Based Biomimetic Nanofibrous Scaffold with Muscle Cells for Hollow Organ Tissue Engineering. ACS Biomaterial Science and Engineering. 2016;2:19-29.

Micol LA, Arenas da Silva LF, Geutjes PJ, Oosterwijk E, Hubbell JA, Feitz WF, et al. In-vivo performance of high-density collagen gel tubes for urethral regeneration in a rabbit model. Biomaterials. 2012:7447-55.

Mikami H, Kuwahara G, Nakamura N, Yamato M, Tanaka M, Kodama S. Two-layer tissue engineered urethra using oral epithelial and muscle derived cells. J Urol. 2012:1882-9.

Nuininga JE, van Moerkerk H, Hanssen A, Hulsbergen CA, Oosterwijk-Wakka J, Oosterwijk E, et al. Rabbit urethra replacement with a defined biomatrix or small intestinal submucosa. Eur Urol. 2003:266-71.

Nuininga JE, Koens MJ, Tiemessen DM, Oosterwijk E, Daamen WF, Geutjes PJ, et al. Urethral reconstruction of critical defects in rabbits using molecularly defined tubular type I collagen biomatrices: key issues in growth factor addition. Tissue Eng Part A. 2010:3319-28.

Olsen L, Bowald S, Busch C, Carlsten J, Eriksson I. Urethral reconstruction with a new synthetic absorbable device. An experimental study. Scand J Urol Nephrol. 1992:323-6.

Orabi H, AbouShwareb T, Zhang Y, Yoo JJ, Atala A. Cell-seeded tubularized scaffolds for reconstruction of long urethral defects: a preclinical study. Eur Urol. 2013:531-8.

Parnigotto PP, Gamba PG, Conconi MT, Midrio P. Experimental defect in rabbit urethra repaired with acellular aortic matrix. Urol Res. 2000:46-51.

Peng WB, Liu CX, Xie Q, Zhu BL, Lv TR. Study on constructing tissue engineered urethra by enriched rabbit hair follicle stem cells. [Chinese]. Journal of Shanghai Jiaotong University (Medical Science). 2013:285-389.

Rotariu P, Yohannes P, Alexianu M, Gershbaum D, Pinkashov D, Morgenstern N, et al. Reconstruction of rabbit urethra with surgisis small intestinal submucosa. J Endourol. 2002:617-20.

Sayeg K, Freitas-Filho LG, Waitzberg AFL, Arias VEA, Laks M, Egydio FM, et al. Integration of collagen matrices into the urethra when implanted as onlay graft. International Braz J Urol. 2013:414-23.

Shokeir A, Osman Y, El-Sherbiny M, Gabr M, Mohsen T, El-Baz M. Comparison of partial urethral replacement with acellular matrix versus spontaneous urethral regeneration in a canine model. Eur Urol. 2003:603-9.

Shokeir A, Osman Y, Gabr M, Mohsen T, Dawaba M, el-Baz M. Acellular matrix tube for canine urethral replacement: is it fact or fiction? J Urol. 2004:453-6.

Sievert KD, Bakircioglu ME, Nunes L, Tu R, Dahiya R, Tanagho EA. Homologous acellular matrix graft for urethral reconstruction in the rabbit: histological and functional evaluation. J Urol. 2000:1958-65.

Sievert KD, Wefer J, Bakircioglu ME, Nunes L, Dahiya R, Tanagho EA. Heterologous acellular matrix graft for reconstruction of the rabbit urethra: histological and functional evaluation. J Urol. 2001:2096-102.

Sun D, Yang Y, Wei Z, Xu Y, Zhang X, Hong B. Engineering of pre-vascularized urethral patch with muscle flaps and hypoxia-activated hUCMSCs improves its therapeutic outcome. J Cell Mol Med. 2014.

Verit A, Darcin OT, Yeni E, Unal D, Ozardali I, Karatas OF. Neointimised ringed polytetrafluoroethylene tubing graft as a prosthesis in the canine urethra: A preliminary experimental study. [Turkish]. Turk Uroloji Dergisi. 2003:241-4.

Villoldo GM, Loresi M, Giudice C, Damia O, Moldes JM, DeBadiola F, et al. Histologic changes after urethroplasty using small intestinal submucosa unseeded with cells in rabbits with injured urethra. Urology. 2013:1380 e1-5.

Wang DJ, Li MY, Huang WT, Lu MH, Hu C, Li K, et al. Repair of urethral defects with polylactid acid fibrous membrane seeded with adipose-derived stem cells in a rabbit model. Connective tissue research. 2015:1-6.

Wang F, Liu T, Yang L, Zhang G, Liu H, Yi X, et al. Urethral reconstruction with tissue-engineered human amniotic scaffold in rabbit urethral injury models. Medical Science Monitor. 2014;20:2430-8.

Wang JH, Xu YM, Fu Q, Song LJ, Li C, Zhang Q, et al. Continued sustained release of VEGF by PLGA nanospheres modified BAMG stent for the anterior urethral reconstruction of rabbit. Asian Pac J Trop Med. 2013:481-4.

Wang YQ, Li YQ, Liu LQ, Xu JJ, Huo R, Li Q, et al. Rabbit urethral defect repair with freeze-dried acellular bladder submucosa. [Chinese]. Zhonghua Zheng Xing Wai Ke Za Zhi. 2005:62-5.

Xie H, Campbell CE, Shaffer BS, Gregory KW. Different outcomes in urethral reconstruction using elastin and collagen patches and conduits in rabbits. J Biomed Mater Res B Appl Biomater. 2007:269-73.

Xie M, Song L, Wang J, Fan S, Zhang Y, Xu Y. Evaluation of stretched electrospun silk fibroin matrices seeded with urothelial cells for urethra reconstruction. J Surg Res. 2013:774-81.

Xie M, Xu Y, Song L, Wang J, Lv X, Zhang Y. Tissue-engineered buccal mucosa using silk fibroin matrices for urethral reconstruction in a canine model. J Surg Res. 2013.

Xu LS, Zhang JM, Zuo ZN, Chu HH. Urethral reconstruction using acellular corpus spongiosum-urethral matrix graft in rats. [Chinese]. Journal of Clinical Rehabilitative Tissue Engineering Research. 2007:3784-6.

Xu Y, Sun DC, Wei ZT, Hong BF, Yang Y. Experimental study on transplantation of autologous minced muscle with human umbilical cord mesenchymal stem cells for urethral reconstruction. European review for medical and pharmacological sciences. 2014;18:3412-9.

Yang SX, Yao Y, Hu YF, Song C, Wang LL, Jin HM. Reconstruction of rabbit urethra using urethral extracellular matrix. Chin Med J (Engl). 2004:1786-90.

Zhang K, Guo X, Zhao W, Niu G, Mo X, Fu Q. Application of Wnt Pathway Inhibitor Delivering Scaffold for Inhibiting Fibrosis in Urethra Strictures: In Vitro and in Vivo Study. International journal of molecular sciences. 2015;16:27659-76.

Zhang Q, Shan Y, Li LP, Fan YZ, Wang JX. Bone marrow mononuclear cells-differentiated vascular endothelial progenitor cells for urethral defect repair in rabbits. Journal of Clinical Rehabilitative Tissue Engineering Research. 2008:8583-7.

Zhang Y, Zhou Y, Jia Ls, Zhai Jm, Miao Xf, Wang R. Role of adipose-derived mesenchynal stem cell combined with silk fibroin scaffold in enhancing vascular formation in rabbit urethral repair. Journal of Clinical Rehabilitative Tissue Engineering Research. 2011:8777-81.

Appendix 4

Reference list on alphabetical order of clinical studies included in Table 2.

Atala A, Guzman L, Retik AB. A novel inert collagen matrix for hypospadias repair. Journal of Urology. 1999:1148-51.

Bhargava S, Patterson JM, Inman RD, MacNeil S, Chapple CR. Tissue-Engineered Buccal Mucosa Urethroplasty-Clinical Outcomes. European Urology. 2008:1263-71.

Carpenter CP, Daniali LN, Shah NP, Granick M, Jordan ML. Distal urethral reconstruction with AlloDerm: a case report and review of the literature. The Canadian journal of urology. 2012:6207-10.

Donkov II, Bashir A, Elenkov CH, Panchev PK. Dorsal onlay augmentation urethroplasty with small intestinal submucosa: Modified Barbagli technique for strictures of the bulbar urethra. International Journal of Urology. 2006:1415-7.

El-Kassaby AW, Retik AB, Yoo JJ, Atala A. Urethral stricture repair with an off-the-shelf collagen matrix. Journal of Urology. 2003:170-3.

El Kassaby A, AbouShwareb T, Atala A. Randomized Comparative Study Between Buccal Mucosal and Acellular Bladder Matrix Grafts in Complex Anterior Urethral Strictures. Journal of Urology. 2008:1432-6.

Fiala R, Vidlar A, Vrtal R, Belej K, Student V. Porcine Small Intestinal Submucosa Graft for Repair of Anterior Urethral Strictures. European Urology. 2007:1702-8.

Fossum M, Skikuniene J, Orrego A, Nordenskjold A. Prepubertal follow-up after hypospadias repair with autologous in vitro cultured urothelial cells. Acta Paediatrica, International Journal of Paediatrics. 2012:755-60.

Glybochko PV, Aljaev JG, Nikolenko VN, Shehter AB, Vinarov AZ, Istranov LP, et al. [TISSUE-ENGINEERED SUBSTITUTION URETHROPLASTY BASED ON DECELLULARIZED VASCULAR MATRIX AND AUTOLOGOUS CELLS OF THE BUCCAL MUCOSA: THE FIRST EXPERIENCE]. Urologiia (Moscow, Russia : 1999). 2015:4-10.

Hauser S, Bastian PJ, Fechner G, Muller SC. Small intestine submucosa in urethral stricture repair in a consecutive series. Urology. 2006:263-6.

Kim JY, Bullocks JM, Basu CB, Bienstock A, Link R, Kozovska M, et al. Dermal composite flaps reconstructed from acellular dermis: a novel method of neourethral reconstruction. Plast Reconstr Surg. 2005:96e-100e.

Le Roux PJ. Endoscopic urethroplasty with unseeded small intestinal submucosa collagen matrix grafts: A pilot study. Journal of Urology. 2005:140-3.

Li P, Li S, Zhao M, Shi B, Li Q, Wang Y, et al. Urethral reconstruction using gelatin sponge and micro-mucosa graft combined with local flap. [Chinese]. Zhongguo xiu fu chong jian wai ke za zhi = Zhongguo xiufu chongjian waike zazhi = Chinese journal of reparative and reconstructive surgery. 2009:313-5.

Lin J, Hao JR, Jin J, Deng SM, Hu J, Na YQ. Homologous dermal acellular matrix graft for urethral reconstruction in man (report of 16 cases). [Chinese]. National Medical Journal of China. 2005:1057-9.

Mantovani F, Trinchieri A, Mangiarotti B, Nicola M, Castelnuovo C, Confalonieri S, et al. Reconstructive urethroplasty using porcine acellular matrix: preliminary results. [Italian]. Arch Ital Urol Androl. 2002:127-8.

Mantovani F, Trinchieri A, Castelnuovo C, Romano AL, Pisani E. Reconstructive Urethroplasty Using Porcine Acellular Matrix. European Urology. 2003:600-2.

Orabi H, Safwat AS, Shahat A, Hammouda HM. The use of small intestinal submucosa graft for hypospadias repair: Pilot study. Arab Journal of Urology. 2013:415-20.

Osman NI, Patterson JM, MacNeil S, Chapple CR. Long-term follow-up after tissue-engineered buccal mucosa urethroplasty. European Urology. 2014;66:790-1.

Palminteri E, Berdondini E, Colombo F, Austoni E. Small Intestinal Submucosa (SIS) Graft Urethroplasty: Short-term Results. European Urology. 2007:1695-701.

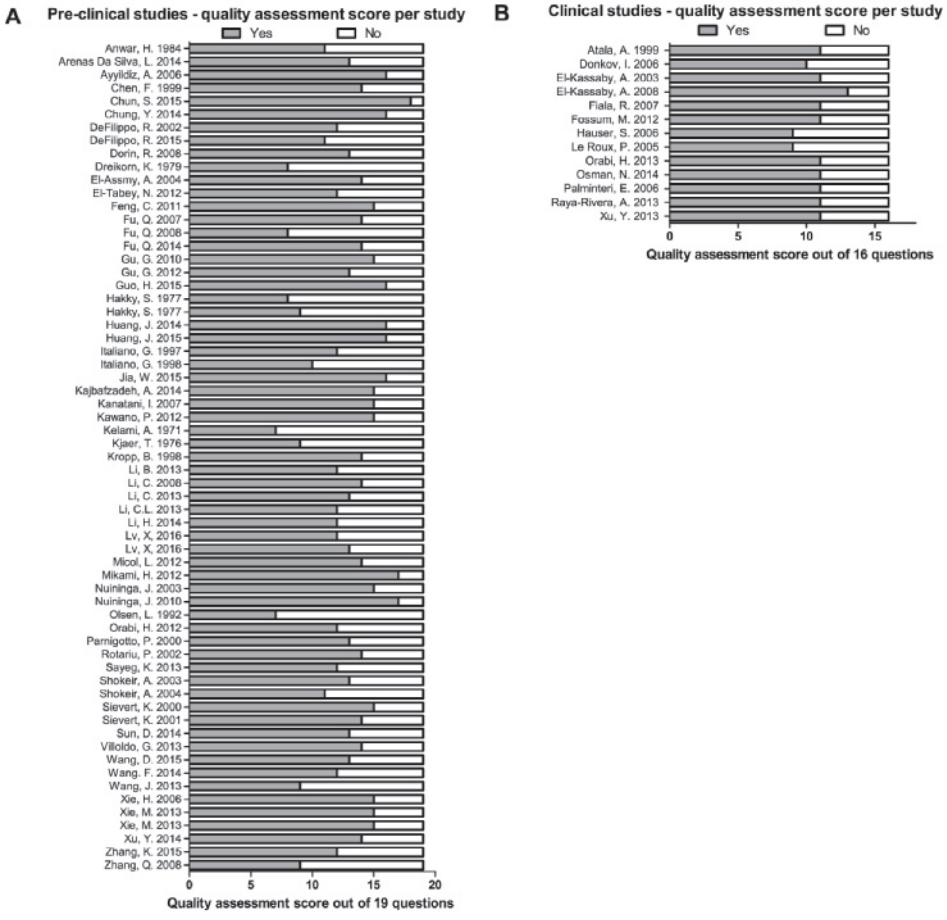
Raya-Rivera A, Esquiliano DR, Yoo JJ, Lopez-Bayghen E, Soker S, Atala A. Tissue-engineered autologous urethras for patients who need reconstruction: An observational study. The Lancet. 2011:1175-82.

Villavicencio H. [Reconstructive plastic surgery of urethral stenosis: urethroplasty with lyophilized dura mater]. Archivos espanoles de urologia. 1989;42:309-12.

Xu YM, Fu Q, Sa YL, Zhang J, Song LJ, Feng C. Outcome of small intestinal submucosa graft for repair of anterior urethral strictures. International Journal of Urology. 2013:622-9.

Yang Wz, Guo Jy, Zhang Yq, Ma T, Li Y, An F. Tissue engineered patch treatment of the urinary leakage after hypospadias repair. Journal of Clinical Rehabilitative Tissue Engineering Research. 2011:7973-6.

Appendix 5



Quality assessment score per study for A) pre-clinical studies and B) clinical studies. Quality was scored based on 19 questions for pre-clinical studies and 17 questions for clinical studies (see Figure 2). Detailed scores per study are available upon request.

Appendix 6

Numerical data on which Figure 3 is based. Estimated probability including 95% confidence intervals for the absence of side effects, functionality and study completion for A) full circumferential repair and B) inlay repair in pre-clinical studies, both categorized for the use of cells and the type of biomaterial. C) For the clinical studies, only decellularized material with or without cells could be analyzed. The effect of cells on the three outcome measures was calculated in estimated probabilities. Overall differences for cellular vs. acellular templates were determined for each outcome measure for both full and inlay repair: * p=0.003, ** p=0.001, all other differences were not significant (p>0.05).

A. Pre-clinical studies – Full circumferential repair

	Point estimate and 95% CI [lower:upper]			
	Biomaterial type	No side-effects	Functionality	Study completion
Acellular	Decellularized	0.04 [0.001:0.46]	0.76 [0.09:0.99]	1.0 [0.63:1.0]
	Natural	0.24 [0.07:0.57]	0.87 [0.04:1.0]	0.95 [0.26:1.0]
	Synthetic	0.80 [0.56:0.93]	0.96 [0.25:1.0]	0.98 [0.49:1.0]
	Overall	0.36 [0.26:0.48]	0.81 [0.14:0.99]	0.98 [0.66:1:0]
Cellular	Decellularized	0.99 [0.68:1.0]	0.77 [0.01:0.99]	1.0 [0.58:1.0]
	Natural	0.73 [0.19:0.97]	1.0 [0.59:1.0]	1.0 [0.52:1.0]
	Synthetic	1.0 [0.00:1.0]	unable to estimate	1.0 [0.03:1.0]
	Overall	0.91 [0.59:0.99]	0.87 [0.07:1.0]	1:0 [0.88:1.0]

B. Pre-clinical studies – Inlay repair

	Point estimate and 95% CI [lower:upper]			
	Biomaterial type	No side-effects	Functionality	Study completion
Acellular	Decellularized	0.49 [0.26:0.73]	0.98 [0.88:1.0]	unable to estimate
	Natural	0.74 [0.04:1.0]	1.0 [0.36:1.0]	unable to estimate
	Synthetic	0.26 [0.22:0.29]	0.73 [0.26:0.95]	unable to estimate
	Overall	0.50 [0.20:0.80]	0.90 [0.66:0.98]	1.0 [0.92:1.0]
Cellular	Decellularized	0.85 [0.03:1.0]	1.0 [0.34:1.0]	unable to estimate
	Natural	1.0 [1.0:1.0]	1.0 [0.42:1.0]	unable to estimate
	Synthetic	0.40 [0.29:0.52]	0.86 [0.31:0.99]	unable to estimate
	Overall	0.75 [0.44:0.92]	0.95 [0.66:0.99]	1:0 [0.75:1.0]

C. Clinical studies – Inlay repair

		Point estimate and 95% CI [lower:upper]		
	Biomaterial type	No side-effects	Functionality	Study completion
Decel.	Acellular	0.70 [0.49:0.85]	0.84 [0.64:0.94]	0.55 [0.18:0.87]
	Cellular	1.0 [0.20:1.0]	0.94 [0.43:1.0]	0.04 [0.002:0.43]

DESIGN OF AN ELASTICIZED COLLAGEN SCAFFOLD:

A method to induce elasticity in a rigid protein

Luuk R. Versteegden*
Henk R. Hoogenkamp*
Roger M. Lomme
Harry van Goor
Dorien M. Tiemessen
Paul J. Geutjes
Egbert Oosterwijk
Wout F. Feitz
Theo G. Hafmans
Nico Verdonschot
Willeke F. Daamen
Toin H. van Kuppevelt
*Contributed equally

Acta Biomaterialia. OCT 2016, volume 44, p277-85.

Abstract

Type I collagen is widely applied as a biomaterial for tissue regeneration. In the extracellular matrix, collagen provides strength but not elasticity under large deformations, a characteristic crucial for dynamic organs and generally imparted by elastic fibers. In this study, a methodology is described to induce elastic-like characteristics in a scaffold consisting of solely type I collagen.

Tubular scaffolds are prepared from collagen fibrils by a casting, molding, freezing and lyophilization process. The lyophilized constructs are compressed, corrugated and subsequently chemically crosslinked with carbodiimide in the corrugated position. This procedure induces elastic-like properties in the scaffolds that could be repeatedly stretched five times their original length for at least 1000 cycles. The induced elasticity is entropy driven and can be explained by the introduction of hydrophobic patches that are disrupted upon stretching thus increasing the hydrophobic-hydrophilic interface. The scaffolds are cytocompatible as demonstrated by fibroblast cell culture.

In conclusion, a new straightforward technique is described to endow unique elastic characteristics to scaffolds prepared from type I collagen alone. Scaffolds may be useful for engineering of dynamic tissues such as blood vessels, ligaments, and lung.

1. Introduction

Insoluble type I collagen is a biomaterial that has been extensively used in the field of tissue engineering and regenerative medicine as scaffolding material. In the native extracellular matrix, collagen provides structural support to tissues. Individual triple helical collagen monomers are densely packed into collagen fibrils that are subsequently organized in bundles called fibers which add strength and prevent the tissue from rupturing upon exposure to mechanical stress. Ideally, in tissue engineering the scaffold should temporarily take over the role of the native extracellular matrix and in this respect type I collagen is often applied. Collagen is widely available from animal sources, easy to purify, biodegradable and well-recognized for its excellent cross-species biocompatibility [1]. In addition, collagen is easy to modify with functional groups, such as glycosaminoglycans or growth factors, and moreover, its biodegradability can be regulated by crosslinking technique [2]. Numerous type I collagen based scaffolds in the field of tissue engineering are already used in the clinical setting showing the value of collagen as a biomaterial [3].

Collagen-based scaffolds generally do not exhibit elastic properties at high deformations, which is of importance for the mechanical compliance of dynamic tissues. Many of these organs, such as lungs, heart valves, ligaments, blood vessels, skin, and bladder, need to be both strong and elastic to prevent ruptures and to reversibly deform [4, 5]. The extracellular matrix of such organs contains elastic fibers, which give the tissue the required resilience and ensure that it regains its original shape after every deformation. Elastic fibers are mainly composed of the protein elastin which is responsible for the elasticity of the tissue [6]. The elastic fibers together with the collagen fibers largely determine the overall mechanical properties of a specific tissue.

In several studies, insoluble elastin-collagen scaffolds have been designed in order to mimic the natural matrix of elastic tissues [7, 8]. However the use of insoluble elastin fibers comes with several disadvantages. A major problem is that elastin usually induces calcification *in vivo*, which may lead to life threatening side-effects [2, 9-11]. Calcification in porcine heart valves for example, is a major clinical problem and has especially been observed in children [12], possibly in line with calcification of elastic fibrils in young animals [13]. Many attempts have been performed to improve clinical applicability including recellularization, surface treatment, enzyme treatment, but the clinical efficacy remains debatable [14]. In addition, the extreme insolubility of elastic fibers complicates the scaffold production process and will further complicate

clinical approval since it is an additional animal derived component. Hydrolyzed or solubilized elastin are easier to handle and have been shown to stimulate synthesis of native elastin fibers [15], but they do not endow the initial scaffolds with major elastic properties. Conferring elasticity to collagen-only scaffolds would therefore be a major advantage in the design of functional scaffolds for tissue engineering of organs/tissues which are subjected to considerable deformations.

In this manuscript, we describe a new method to induce elasticity in scaffolds consisting of only type I collagen fibrils, thereby circumventing the issues that are associated with the use of elastin. By compression and corrugation of lyophilized porous collagen constructs followed by carbodiimide crosslinking under physical restraint, we were able to alter the morphology, and induce elastic-like, entropy-driven, characteristics. The combination of both strength and elastic-like properties in a one-component scaffold offers new opportunities for tissue engineering of dynamic tissues.

2. Materials and methods

2.1. Type I collagen fibrils

Highly-purified type I collagen fibrils were obtained as previously described [16]. Briefly, bovine achilles tendons were pulverized under liquid nitrogen-cooled conditions using a universal cutting mill (Pulverisette19, Fritsch GmbH, Idar-Oberstein, Germany) with a sieve-opening of 0.5 mm. The purification process included washings with aqueous solutions of NaCl (1.0 M), urea (6.0 M), and acetic acid (0.25 M), acetone and demineralized water.

2.2. Scaffold construction

2.2.1. Preparation of a porous collagen scaffold

A 0.8% (w/v) collagen suspension was prepared by mixing purified type I collagen fibrils with 0.25 M acetic acid. This suspension was swollen overnight and homogenized using a Silverson L5M-A laboratory mixer (Silverson, Chesham, UK) by mixing 3 min at 2,500 rpm using a general purpose disintegrating workhead, followed by 3 min at 2,500 rpm with a slotted workhead. The suspension was deaerated using centrifugation at 100 g. All steps were performed at 4°C to prevent denaturation of the collagen. The collagen suspension was poured into a 10 mL polypropylene mold with a 6 mm stainless steel (grade 304) mandrel inside and frozen for at least 4 h at -20°C in aluminum freezing blocks. After removal of the mandrel the frozen constructs were lyophilized (Zirbus sublimator 500II, Bad Grund, Germany).

2.2.2. Preparation of compressed and corrugated collagen scaffolds

After freeze-drying, the porous constructs (P-tube), with the mandrels again put in place, were uniformly compressed around the mandrel by gently squeezing them between two plane surfaces under a rolling motion to create porous compressed scaffolds (PC-tube, Figure 1A). Next, in order to create porous compressed and corrugated scaffolds (PCC-tube), two discs fitting exactly over the mandrels were positioned around each side of the scaffold and subsequently pushed together until 10 mm space was left in between (Figure 1A). The discs were replaced by 6 mm rubber O-rings to hold the scaffold in its corrugated state. Next, the scaffolds were crosslinked using a zero-length crosslinking method applying EDC (Merck Schuchardt OHG, Hohenbrunn, Germany) and NHS (Fluka Chemie AG, Buchs, Switserland) [17]. In brief, scaffolds were crosslinked for 3 h at room temperature in 50 mM 2-morpholinoethane sulphonic acid (MES buffer, pH 5.0) (USB, Ohio, USA) containing 40% (v/v) ethanol, 33 mM EDC and 6 mM NHS and subsequently washed in 0.1 M Na_2HPO_4, (2x), 1 M NaCl (2x), 2 M NaCl (4x), and demineralized water (6x). Finally, scaffolds were placed in 70% (v/v) ethanol and stored at -20°C until use.

2.3. Scaffold Characterization

2.3.1. Macroscopic evaluation and video recording

Macroscopic images were taken and videos were recorded using a Canon 1d X with a EF macro lens camera (Canon, Melville, NY, USA). High-speed videos (1000 frames per second) were recorded using a Casio EX-ZR100 (Casio, Tokyo, Japan) with camera speed settings at HS1000 and resolution at 224x64 pixels.

2.3.2. Scanning electron microscopy

Scanning electron microscopy(SEM) was used to analyze the morphology and structure of the tubular scaffolds. Samples were lyophilized, fixed on a stub with double-sided carbon tape and sputtered with an ultrathin gold layer in a Polaron E5100 Coating System. Examination was performed in a JEOL SEM 6310 apparatus (JEOL Ltd, Tokyo, Japan) with an accelerating voltage of 10 kV.

2.3.3. Effect of fluids with different polarities on elastic-like characteristics

PCC-tubes were restrained in a stretched position and air-dried. Next, the stretched scaffolds were placed in fluids of different polarities (*i.e.* water, ethylene glycol,

methanol, ethanol, 1-propanol, 1-butanol, acetone and chloroform (all from Sigma-Aldrich) [18]. Before use, non-polar fluids were dehydrated by adding anhydrous Cu(II)SO_4 crystals (Sigma-Aldrich). The ability of the stretched scaffold to return to its original corrugated state was measured as a percentage of the maximal corrugation (corrugation in water was set to 100%). This experiment was performed three times independently. In addition, to visualize the influence of polarity of the fluid on the ability of the scaffold to return to its corrugated position, an experimental set up combining water and chloroform was applied. An air-dried stretched scaffold was placed in a tube and chloroform was added until half of the scaffold was submerged. Next, water with Cu(II)SO_4 (to increase the contrast between the two layers) was added on top of the chloroform and the scaffold was monitored over time. Images were taken using a Sony Cyber-shot DSC-H10.

2.3.4. Mechanical properties

To characterize the mechanical properties of the scaffolds, force-monitored ultimate tensile strength experiments were performed and force-displacement curves were determined by using a Zwick/Roell Z2.5 testing machine (Zwick/Roell, Ulm, Germany). From these curves ultimate tensile strength was derived. In addition, the work needed for break was determined by calculating the area under the curve. Curves were converted into force-strain curves in which strain was calculated as percentage of the original scaffold length after crosslinking. Tests were performed by clamping 2 mm of the scaffolds between two custom made clamps (Supplementary Figure 1) and pulling them apart in opposite direction with 50 mm $min.^{-1}$ (n=19). For fatigue tests, corrugated scaffolds were stretched and released 1,000 times with a speed of 167 mm $min.^{-1}$ over a distance at which the corrugated structure was totally unfolded while continuously measuring the force (n=4). Scaffolds were wetted with 0.1 M phosphate buffered saline (pH 7.4) using a peristaltic pump. Data was processed using TestXpert II V3.5 software (Zwick/Roell).

2.3.5. Evaluation of crosslinking by assaying primary amine groups

The degree of crosslinking of P, PC and PCC-tubes was evaluated by the loss of primary amine groups upon crosslinking as measured by a trinitrobenzene sulfonic acid assay [7]. In brief, freeze-dried samples were incubated for 30 min. in 4% (w/v) $NaHPO_4$ (Merck, Darmstadt, Germany) at room temperature. Next, 0.5% (w/v) 2,4,6-trinitrobenzesulfonic acid (Fluka Chemie AG, Buchs, Switzerland) was added and samples were incubated for 2 h at 40°C followed by a hydrolysis step with 6 M HCl for 1.5 h at 60 °C. Demineralized water was added to dilute the samples before they were measured at 420 nm with a

spectrophotometer (Bio-Tek, Bad Friedrichshall, Germany). Glycine (Scharlau Chemie, Barcelona, Spain) was used for the calibration curve. Analysis was in triplicate in three independent experiments.

2.4. In vitro evaluation

2.4.1. Cell culture

NIH/3T3 fibroblasts (ATCC, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's Medium Glutamax (Life Technologies, Carlsbad, CA, USA) containing 10% (v/v) fetal bovine serum (GE Healthcare, PAA Laboratories, Pasching, Austria), penicillin (1,000 IU mL^{-1}) and streptomycin (1,000 μg mL^{-1}, PAA Laboratories) at 37°C under an atmosphere containing 5% CO_2. Confluent cells were harvested using 0.25% (w/v) trypsin-EDTA (Life Technologies).

2.4.2. Cell seeding on scaffolds

For cell seeding experiments, scaffolds were cut into strips of 0.5 x 2 cm (P-tube and PC-tube), and 0.5 cm x 1 cm for the PCC-tube. The strips were disinfected by six washing steps with 70% (v/v) ethanol including one overnight step. To remove ethanol, six washings with sterile 0.1 M phosphate buffered saline, pH 7.4 (PBS, Braun, Melsungen, Germany) were performed with one overnight step. Finally, strips were incubated in culture medium for 1 h prior to cell seeding. The strips were transferred to a 6-well suspension plate and 4.5 x 10^5 cells in 0.75 mL were added to each scaffold. After 3h, 3.25 mL of culture media was added. Constructs were cultured for four days and media was renewed every other day.

2.4.3. Alamar Blue cell viability assay

To investigate the cell viability of the 3T3 fibroblasts seeded on the scaffold, an Alamar Blue assay (Life Technologies, Carlsbad, CA, USA) was performed. For each scaffold type, 6 mm punches were taken and subsequently placed in 96-wells plates. Next, 1.0 x 10^4 cells in 25 μL were seeded in each well in triplicate and after three hours 200 μL culture media was added. Alamar Blue was added 4, 24 and 96 hours after seeding. Fluorescence was measured using an excitation wavelength of 540 nm and an emission wavelength 620 nm. This experiment was performed three times independently.

2.4.4. Histological evaluation of scaffolds

For histology, constructs were embedded in Tissue-Tek (Sakura, Torrance, USA) and frozen in dry-ice cooled 2-methylbutane (Sigma-Aldrich, St. Louis, USA). Next, they

were sectioned (5 μm) using a cryostat microtome (Heidelberg, Heidelberg, Germany), mounted on superfrost slides (Thermo Scientific, Menzel GmbH & Co KG, Braunschweig, Germany) and stored at -20 °C until use. Before staining, fixation with cold acetone (-20 °C) was applied for 15 min. Sections were stained with haematoxylin and eosin (HE), DAPI (nuclear stain) and immunostained for type I collagen. For the latter, sections were blocked for 15 min. in 1% (v/v) BSA in PBS, pH 7.4, and subsequently incubated for 45 min. with a rabbit-anti-bovine type I collagen antibody (dilution 1:250, Millipore, Cambridge, UK) followed by 3 x 5 min. washings with PBS. Bound antibodies were visualized by incubation for 45 min. using Alexa Fluor 594-conjugated goat-anti-rabbit IgG (dilution 1:250, Invitrogen, Eugene, OR, USA). For nuclear staining, section were washed 3 x 5 min. with PBS and stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, 10 μg mL^{-1} in PBS, Sigma Aldrich, St. Louis MO, USA). After extensive washings with PBS, cover slips were mounted using Mowiol (Calbiochem, San Diego, CA, USA). Sections were analyzed using a Leica CTR6000 microscope (Leica Microsystems GmbH, Wetzlar, Germany).

2.5. Statistical analysis

Statistical analysis on all data was performed in GraphPad PRISM 5, version 5.03 (GraphPad Software Inc, La Jolla, CA, USA). For the mechanical characterization (section 2.3.4.) and the TNBS-assay (section 2.3.5.) groups were compared using a one-way ANOVA with Bonferroni post-hoc tests. For the Alamar Blue cell viability (section 2.4.3.) the differences for P-, PC- and PCC-tube between each time-point were analyzed using a one-way ANOVA with Bonferroni post-hoc tests. Differences between the scaffolds within one time-point were analyzed with a two-way ANOVA with Bonferroni post-hoc test.

3. Results

3.1. Scaffold morphology and degree of crosslinking

Swelling, homogenization, freezing and freeze-drying of bovine collagen resulted in porous tubular scaffolds, which were comparable to scaffolds previously described in literature (Figure 1B-D) [19, 20]. The freeze-dried porous tubular scaffold (P-tube) was used as a starting point to produce collagen scaffolds with elastic-like characteristics. Compressing this porous scaffold around the mandrel resulted in a dense tube with a more compact and longitudinal porosity (PC-tube, Figure 1E-G). Corrugation of this compressed PC-tube tube led to a tubular scaffold a pleated/corrugated wall structure

with compact porosity (PCC-tube, Figure 1H-J). Chemical crosslinking of the scaffolds *in situ* resulted in fixation of the morphology. The degree of crosslinking was determined by measuring the content of free amine groups. Non-crosslinked collagen showed an average of 247±15 nmol of free amine groups per mg collagen, whereas crosslinked P-tube, PC-tube and PCC-tube had averages of 128±17, 102±14 and 112±16 nmol free amine groups per mg collagen respectively, corresponding to a reduction of free amine groups of approximately 48, 59 and 55% (Table 1). PC-tube and PCC-tube were statically different from the P-tube. Between PC and PCC tubes no statistical differences were observed.

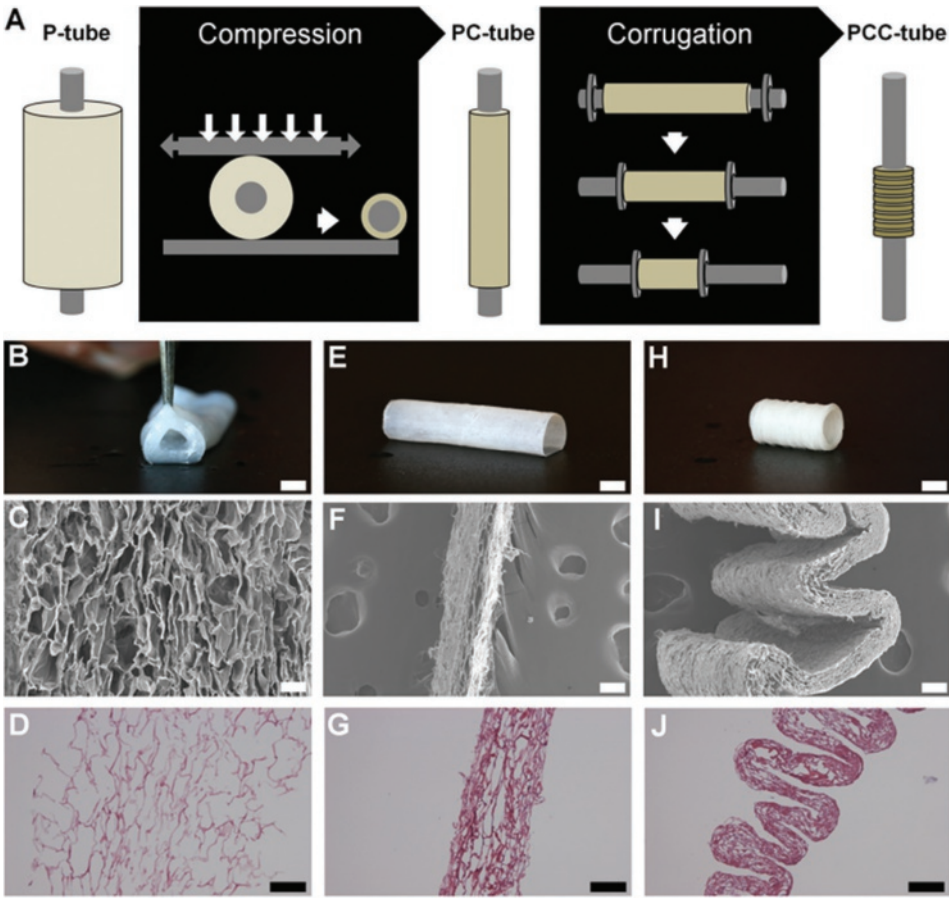


Figure 1. Scaffold preparation and morphological characteristics. A) Schematic representation of the method used to construct a porous compressed tube (PC-tube) out of a porous tube (P-tube), and porous compressed and corrugated tube (PCC-tube) out of a PC-tube. B-D) Macroscopic image, SEM image and H&E stained image of the P-tube, where the porous morphology is clearly seen. E-G) Same images for the PC-tube, where compressed pore structure is visible. H-J) Images of PCC-tube where the corrugated structure of the scaffold is apparent. Scale bars: Macroscopic image = 5 mm, SEM = 100 μm, H&E = 200 μm.

3.2. Mechanical characteristics

Only the PCC-tube scaffolds displayed reversible elastic-like behavior upon stretching. Scaffolds could be elongated, and did return to their relaxed corrugated position upon release of the force (Figure 2A-D and Movie 1). The initial elongation/unfolding of the PCC-tube scaffolds did only cost marginal effort. The mechanical properties of the scaffolds were analyzed using a custom-made clamp system, which could hold the entire tubular scaffold during a force-monitored tensile test (Figure A.1). During tensile testing, the three scaffolds displayed very distinct mechanical properties as seen by the shape of the force-strain curves (Figure 2E). The PCC-tube scaffold did not need a large force (0.1 N) in order to be extended up to about 5 times its length. On average the PCC-tube could be elongated most until rupture (675 ± 46% of the length in the relaxed state), followed by P-tube (126 ± 4%) and PC-tube (117 ± 3%) (Figure 2F). The PC-tube was significantly stronger (4.2 ± 1.1 N) than PCC-tube (3.4 ± 1.0 N) and P-tube (1.8 ± 0.5N) (Figure 2G). Work needed for break (or area under the force-displacement curve) showed that the PCC-tube required significantly more work until scaffold failure (36 ± 8.6 mJ) than PC-tube (24 ± 9.2 mJ) and P-tube (16 ± 5.7 mJ) (Figure 2H). The persistence of elastic-like behavior of the PCC-tube was tested using a fatigue test where the scaffolds were subjected to 1,000 cycles of elongation up to a defined distance whilst recording the force (Figure 2I). The fatigue curve showed an initial decline of tensile force from approximately 0.4 N to 0.37 N in the first 200 cycles, corresponding to a decline of 1.5×10^{-4} N cycle^{-1}. In the following 800 cycles, the approximate rate of decline was only 3.0×10^{-5} N cycle^{-1} (0.37 N to 0.346 N). The fatigue curve graph is derived from the averages of the peak force values of every cycle from four independent measurements as depicted in figure 2J which is a zoomed in view of figure 2I from cycle 800 to 810.

3.3. Mechanism underlying elastic-like properties

The mechanism behind the elastic-like behavior was investigated using liquids with different relative polarities. In the experiment, the PCC tube was air-dried in a stretched instead of a corrugated state. When the PCC-tube was air-dried in a stretched state, it remained in this position. Upon rehydration in water, it returned to its original corrugated length (Figure 3A and Movie 2). Without crosslinking this phenomenon was not observed. When liquids with lower polarities than water were tested in this scenario, it was observed that a decrease in relative polarity prevented the return of the PCC-tube to the corrugated state (Figure 3A-B). To elaborate on this phenomenon, a test was designed where the PCC-tube dried in the elongated state was placed in a

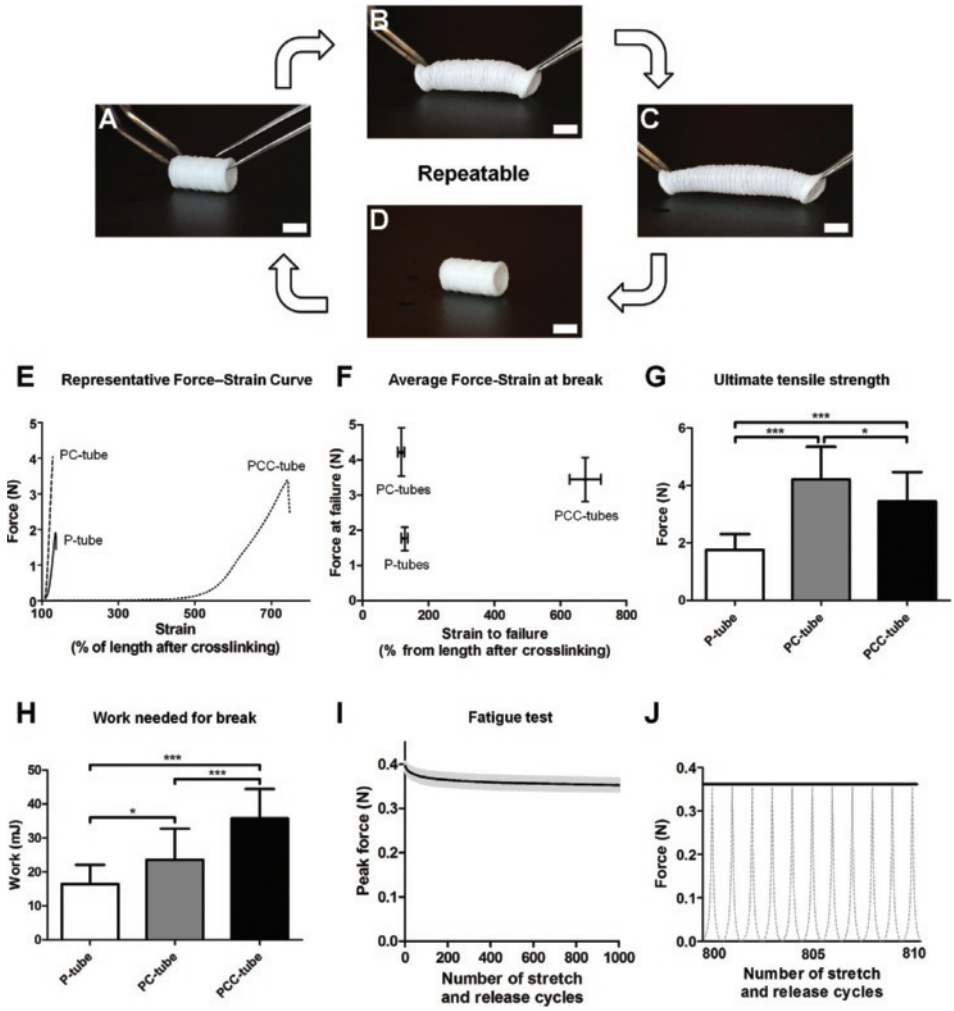


Figure 2. Mechanical characterization of porous (P)-tube, Porous compressed (PC)-tube and porous compressed and corrugated (PCC)-tube scaffolds. A) PCC-tube in relaxed position. B) PCC-tube in semi-extended position. C) PCC-tube in fully extended position. D) PCC-tube again in relaxed position after applied force is released. E) Representative force-strain graph of P-tube, PC-tube, PCC-tube, with differences in extendibility indicated. F) Average force and strain at failure for P-tube, PC-tube and PCC-tube (n=19 per scaffold type) showing the range at which the scaffolds fail. G-H) Ultimate tensile strength and work (area under force-displacement graph) for the three scaffold types derived from force-distance graphs (not shown here), n=19; One-way ANOVA with Bonferroni post-hoc test, * = P < 0.05, ** P < 0.01, *** P < 0.001. I) Fatigue test of PCC-tube, showing persistence of elastic characteristic for at least 1000 stretch and release cycles (n=4). J) Zoomed-in area of a representative part of the force graph from which peak forces per cycle were determined. Scale bar in A-D = ~5 mm.

test-tube with two liquids of different polarity (water and chloroform). Since the liquids are immiscible, they formed a sharp barrier which was visualized by staining water with $Cu(II)SO_4$ [21]. The scaffold was placed in the chloroform layer after which the water was added on top. The part of the PCC-tube scaffold protruding in the water layer returned to the corrugated state, whereas the PCC-tube segment in the chloroform

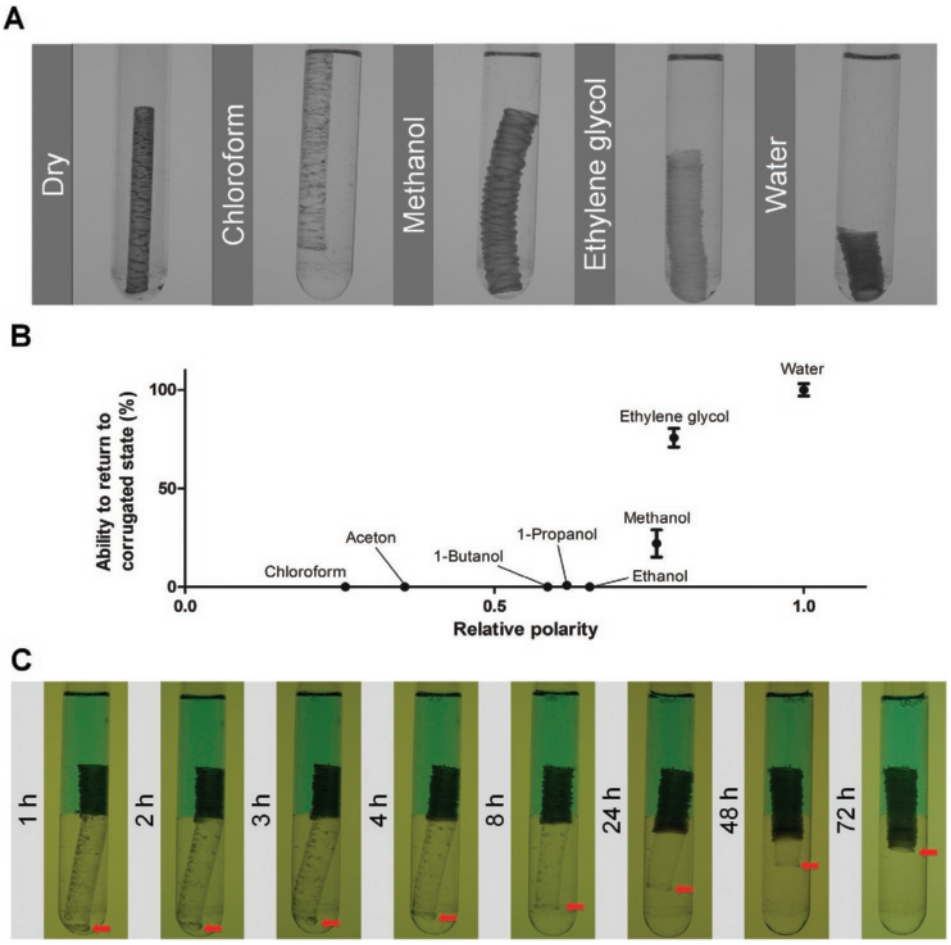


Figure 3. Mechanism of elastic-like behavior of the porous, compressed and corrugated (PCC) tube scaffold. A) Stretched PCC-tubes were air-dried (left) and exposed to fluids with various polarity. At low polarity, the PCC tube scaffold did not return to its original corrugated state. B) Effect of polarity of fluid on the ability of air-dried PCC-tube to return to the original corrugated state (n=3). C) Time-lapse of an stretched air-dried PCC-tube in a two layer system with chloroform (bottom half, colorless) and water (top half, light blue), showing that the part of scaffold in contact with water returned to the original corrugated state, while the part exposed to chloroform remained stretched. Over time the scaffold becomes hydrated and migrated towards the aqueous layer. Red arrow indicates the base of the scaffold.

remained stretched. Over time, the PCC-tube attracted water, with a part of the PCC-tube protruding in the chloroform layer (Figure 3C). This is likely due to the hydrated scaffold being more dense than water.

3.4. Cytocompatibility of scaffolds

To study the effect of compression and corrugation on cells, a viability assay was performed. Cells seeded on PC- and PCC-tubes were able to proliferate to the same extent as cells without compression and corrugation. The number of cells increased in time (Figure 4A). Histological evaluation (Figure 4B-D) further confirmed the outcome the proliferative capacity of cells as indicated by the increase in number of cells compared to the time of seeding. Compression and corrugation resulted in a more closed surface and consequently cells seeded on PC- and PCC-tubes did not penetrate as deep as those seeded on P-tubes. Cells formed large sheets on top of the scaffold resulting in local higher cell densities.

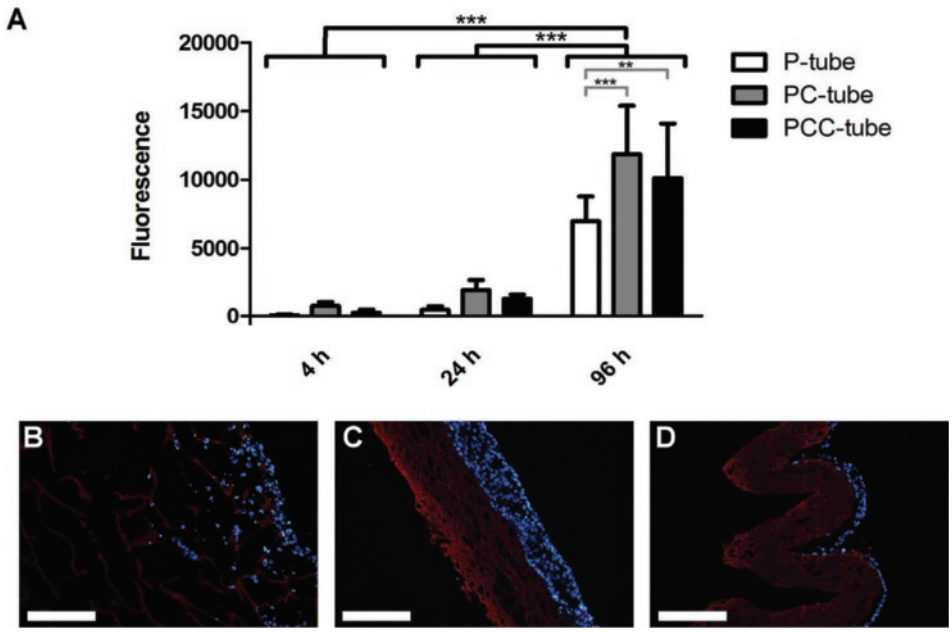


Figure 4. Cytocompatibility of porous (P)-tube, porous, compressed (PC)-tube and porous, compressed and corrugated (PCC)-tube. A) Alamar blue cell viability assay at 4, 24 and 96 h after cell seeding (one way ANOVA with Bonferroni post-hoc test). Differences within time points were analyzed using a two-way ANOVA with Bonferroni post-hoc test. * = $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. B-D) Microscopical images of a cross-section of P-tube B), PC-tube C) and PCC-tube D) cultured for 4 days with fibroblasts, and stained for type I collagen (red) and cell nuclei (blue). Bar = 200 μm.

4. Discussion

4.1. Scaffold characteristics

Methods to modulate the mechanical properties of porous collagen scaffolds have been extensively studied [17, 22-24], focusing on increasing the overall strength either by chemical modification, by introduction of additional components such as synthetic polymers [22, 25] or by compressing porous collagen scaffolds [20, 26]. In this manuscript we have modulated collagen in such a way that new mechanical properties arise. A combination of compression, corrugation and chemical crosslinking under physical restraint resulted in the introduction of elastic characteristics in collagen-only constructs. In this way, the collagen adopted properties of another component of the extracellular matrix i.e. elastin. A fatigue test (1000 cycles of folding-unfolding) indicated the elastic behavior to be durable.

4.2. Proposed mechanism for elasticity

The PCC-tube scaffold has elastic-like properties where the scaffold can be elongated and returns to its relaxed state. It is unlikely the rigid collagen fibrils themselves display elastic properties since they are relatively inelastic [27]. The elastic-like properties of corrugated collagen fibrils in native artery tissue has been previously described, where the corrugated fibrils contribute to the elasticity of the arteries [27]. However, the mechanism behind the phenomenon of an elasticized collagen scaffold (PCC-tube) has, to the best of our knowledge, not been described in previous literature.

Several dedicated experiments were designed to elucidate the mechanism behind the elastic-like properties. The scaffold displayed elastic-like properties when it was wetted, but not when it was dried, a characteristic similar to native elastic fibers. Applying fluids with varying polarity influenced the elastic-like behavior: the less polar the fluid, the less capacity of a dry scaffold to return to its corrugated state. Finally, without crosslinking no elastic behavior was observed.

Taking these data into account, the following mechanism for the elastic-like properties of collagen is proposed (Figure 5). Compression and subsequent corrugation during carbodiimide crosslinking results in the creation of hydrophobic regions at places where the collagen fibrils are densely packed together. The crosslinking process will reduce the amount of free (charged) amine and carboxylic groups (by the formation of amide bonds), thus reducing the overall hydrophilicity of the collagen and increasing

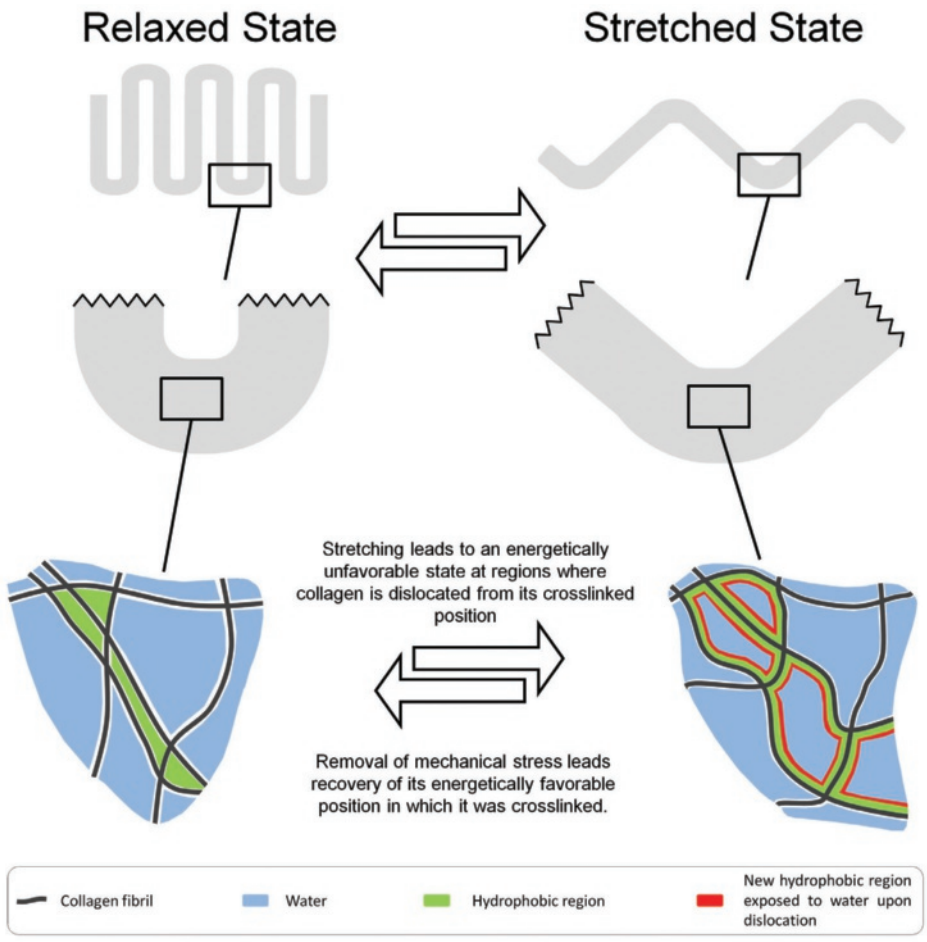


Figure 5: Proposed molecular mechanism of elastic-like properties of porous, compressed and corrugated tube scaffolds. Crosslinking of scaffolds in the corrugated state introduces hydrophobic areas due to loss of free amine and carboxylic groups. Upon stretching hydrophobic areas become exposed to the aqueous environment resulting in an increase of thermodynamically unfavorable interfaces. Upon release of the mechanical stress the scaffolds return to the original corrugated state.

the hydrophobicity. At locations where fibrils are densely packed thermodynamically favored hydrophobic regions are formed which stabilize the corrugated shape. Once tensile force is applied (stretching) the hydrophobic areas will be exposed to the aqueous surroundings, which is thermodynamically unfavorable (Figure 5). When the force is released (relaxation) an entropy-driven force returns the scaffold to the corrugated shape in which the scaffold was crosslinked. Thus, stretching of the fibrils in the scaffold increases the interface between water molecules and the hydrophobic

areas associated with the fibrils, a process that results in more – energetically unfavorable – ordering of water molecules (i.e. loss of entropy). The proposed mechanism resembles entropy-driven mechanism associated with the elasticity of elastin [28-30].

4.3. Cytocompatibility

Collagen is a highly characterized biomaterial and the cellular response to collagen-based biomaterials is generally well understood [31-33]. However, modification of the collagen structure or chemical make-up can influence the cellular response [34]. In the case of the compressed (PC-tube) and corrugated (PCC-tube) scaffolds, the effect of compression, corrugation and subsequent crosslinking was unknown. In this respect it was necessary to determine the biocompatibility of the constructs after the processing steps. In the PC-tube and PCC-tube cells did not penetrate the scaffold and appear to form multilayer sheet-like structures on top of the scaffold.

Cells can adhere directly to collagen via e.g. α1β1 integrins, or indirectly via $\alpha_v\beta3$ and α5β1 integrins to fibronectin present in culture medium [35, 36]. Carbodiimide crosslinking, as used in this study, may affect binding sites for integrins on collagen [37], and therefore initial binding of fibroblasts to collagenous scaffolds may have been through serum-derived fibronectin.

In this study, it was shown that after 96 h of culturing, both PC-tube and PCC-tube scaffolds seem to contain an equal or higher amount of viable cells compared to P-tube scaffolds. As the biocompatibility of the P-tube has been extensively studied in both *in vitro* and *in vivo* studies, it is a suitable benchmark for the PC-tube and PCC-tube [38-41].

Overall, the cytocompatibility of the PC-tube and PCC-tube seems to be comparable to the P-tube indicating that the compression, corrugation and subsequent crosslinking do not negatively affect the intrinsic biocompatibility of collagen.

4.4. Implications and applications

To the best of our knowledge, no studies have been reported where a scaffold consisting solely of collagen is given elastic-like characteristics. Other approaches to provide collagen-based scaffolds with elasticity mainly involve the addition of other components, like (recombinant or natural) elastin, silk and synthetic polymers [42, 43]. Using elasticized collagen-only materials may circumvent the use of elastin or

other elastic components and may aid in the straightforward development of scaffold for the repair of dynamic tissues. By varying the extent of crosslinking, compression and corrugation various degrees of elasticity may be obtained in line with the characteristics of tissues that undergo repeated elastic deformation like the blood vessels, ligaments, bladder, skin, and the gastrointestinal tract [44-46]. For instance, a scaffold for bladder repair should be able to expand many times from its contracted size without a significant increase in pressure, subsequently contract to release the urine and repeat this micturition cycle over and over again [46]. The elasticity in the PCC-tube is primarily oriented in a longitudinal direction. It should be noted that for tissues such as blood vessels or the gastrointestinal tract the elasticity is orientated in a circumferential direction to enable the diameter to expand. With an adaptation of the methodology developed it may be possible to meet these requirements and create a collagen scaffold that is in compliance with the elasticity of these tissue.

5. Conclusion

Collagen, generally known for providing strength and rigidity to tissues, was given elastic-like characteristics by combining compression, corrugation and crosslinking. The induced elasticity is likely entropy-driven and the procedure used does not reduce the cytocompatibility of the collagen. The technology may be useful for the construction of scaffolds for regenerative medicine of dynamic organs with intrinsic elasticity.

6. Acknowledgements

The Dept. of Surgery (Radboud university medical center, Nijmegen, Netherlands) is thanked for the use of the Zwick/Roell mechanical testing apparatus and subsequent support. Additionally, technical support received from Leon Driessen of the Dept. of Orthopedics (Radboud university medical center, Nijmegen, Netherlands) is acknowledged. We thank the Technical Support Group (Norbert Hermesdorf, Mark van de Hei and Twan de Bruin) from the Radboud University Faculty of Social Sciences for help with the design and manufacturing of the custom clamp. This study was financially supported by the EU-FP6 project EuroSTEC (soft tissue engineering for congenital birth defects in children, contract: LSHB-CT-2006-037409) and by PIDON (NovioTissue project), a combined subsidiary program of the Dutch Ministry of Economic Affairs, and the states of Gelderland and Overijssel (PID101020). The sources of funding have had no other involvement in this publication.

7. Supplemental Information

Supplemental information is available at https://doi.org/10.1016/j.actbio.2016.08.038.

Table 1. Degree of crosslinking

Scaffold	Amime groups [nmol / mg collagen] ± SD	Reduction of free amine groups	n
Non-crosslinked	247 ± 15	-	9
P-tube	128 ± 17	48 %[1]	27
PC-tube	102 ± 14	59 %[2]	27
PCC-tube	112 ± 16	55 %[2]	27

[1] Statistically different from PC-tube (P<0.001) and PCC-tube (P<0.01), one-way ANOVA with Bonferroni post-hoc test.

[2] Difference between PC-tube and PCC-tube was not significant, one-way ANOVA with Bonferroni post-hoc test.

8. References

[1] L.B. Rocha, G. Goissis, M.A. Rossi, Biocompatibility of anionic collagen matrix as scaffold for bone healing, Biomaterials 23 (2002) 449-56.

[2] C.H. Lee, A. Singla, Y. Lee, Biomedical applications of collagen, International Journal of Pharmaceutics 221 (2001) 1-22.

[3] R. Parenteau-Bareil, R. Gauvin, F. Berthod, Collagen-Based Biomaterials for Tissue Engineering Applications, Materials 3 (2010) 1863.

[4] B. Vrhovski, A.S. Weiss, Biochemistry of tropoelastin, Eur J Biochem 258 (1998) 1-18.

[5] K.J. Aitken, D.J. Bagli, The bladder extracellular matrix. Part I: architecture, development and disease, Nat Rev Urol 6 (2009) 596-611.

[6] A.J. Bruce Alberts, Julian Lewis, Martin Raff, Keith Roberts, and Peter Walter, Molecular Biology of the Cell, 4th edition, 4th ed., Garland Science, New York, 2002.

[7] L. Buttafoco, P. Engbers-Buijtenhuijs, A.A. Poot, P.J. Dijkstra, W.F. Daamen, T.H. van Kuppevelt, I. Vermes, J. Feijen, First steps towards tissue engineering of small-diameter blood vessels: preparation of flat scaffolds of collagen and elastin by means of freeze drying, J Biomed Mater Res B Appl Biomater 77 (2006) 357-68.

[8] A.J. Ryan, F.J. O'Brien, Insoluble elastin reduces collagen scaffold stiffness, improves viscoelastic properties, and induces a contractile phenotype in smooth muscle cells, Biomaterials 73 (2015) 296-307.

[9] M. Wu, C. Rementer, C.M. Giachelli, Vascular calcification: an update on mechanisms and challenges in treatment, Calcif Tissue Int 93 (2013) 365-73.

[10] N.J. Vardaxis, M.E. Boon, J.M. Ruijgrok, Calcification of cross-linked collagen-elastin membrane implants in vivo and their proposed use in bone regeneration, Biomaterials 17 (1996) 1489-97.

[11] M.T. Bailey, S. Pillarisetti, H. Xiao, N.R. Vyavahare, Role of elastin in pathologic calcification of xenograft heart valves, J Biomed Mater Res A 66 (2003) 93-102.

[12] P.R. Cipriano, M.E. Billingham, P.E. Oyer, L.M. Kutsche, E.B. Stinson, Calcification of porcine prosthetic heart valves: a radiographic and light microscopy study, Circulation 66 (1982) 1100-4.

[13] W.F. Daamen, S.T.M. Nillesen, T. Hafmans, J.H. Veerkamp, M.J.A. van Luyn, T.H. van Kuppevelt, Tissue response of defined collagen–elastin scaffolds in young and adult rats with special attention to calcification, Biomaterials 26 (2005) 81-92.

[14] M. Namiri, M.K. Ashtiani, O. Mashinchian, M.M. Hasani-Sadrabadi, M. Mahmoudi, N. Aghdami, H. Baharvand, Engineering natural heart valves: possibilities and challenges, J Tissue Eng Regen Med (2016).

[15] W.F. Daamen, J.H. Veerkamp, J.C.M. van Hest, T.H. van Kuppevelt, Elastin as a biomaterial for tissue engineering, Biomaterials 28 (2007) 4378-4398.

[16] J.S. Pieper, A. Oosterhof, P.J. Dijkstra, J.H. Veerkamp, T.H. van Kuppevelt, Preparation and characterization of porous crosslinked collagenous matrices containing bioavailable chondroitin sulphate, Biomaterials 20 (1999) 847-58.

[17] L.H. Olde Damink, P.J. Dijkstra, M.J. van Luyn, P.B. van Wachem, P. Nieuwenhuis, J. Feijen, Cross-linking of dermal sheep collagen using a water-soluble carbodiimide, Biomaterials 17 (1996) 765-73.

[18] C. Reichardt, Solvents and Solvent Effects in Organic Chemistry, 3rd ed., Wiley-VCH Verlag GmbH & Co. KGaA2003.

[19] H.R. Hoogenkamp, M.J. Koens, P.J. Geutjes, H. Ainoedhofer, G. Wanten, D.M. Tiemessen, J. Hilborn, B. Gupta, W.F. Feitz, W.F. Daamen, A.K. Saxena, E. Oosterwijk, T.H. van Kuppevelt, Seamless vascularized large-diameter tubular collagen scaffolds reinforced with polymer knittings for esophageal regenerative medicine, Tissue Eng Part C Methods 20 (2014) 423-30.

[20] M.J. Koens, K.A. Faraj, R.G. Wismans, J.A. van der Vliet, A.G. Krasznai, V.M. Cuijpers, J.A. Jansen, W.F. Daamen, T.H. van Kuppevelt, Controlled fabrication of triple layered and molecularly defined collagen/elastin vascular grafts resembling the native blood vessel, Acta Biomater 6 (2010) 4666-74.

[21] W.M. Haynes, Handbook of Chemistry and Physics, 92nd Edition, CRC Press, Boca Raton, Florida, USA2011.

[22] T. Bou-Akl, R. Banglmaier, R. Miller, P. VandeVord, Effect of crosslinking on the mechanical properties of mineralized and non-mineralized collagen fibers, J Biomed Mater Res A 101 (2013) 2507-14.

[23] L.E. Freed, G.C. Engelmayr, Jr., J.T. Borenstein, F.T. Moutos, F. Guilak, Advanced material strategies for tissue engineering scaffolds, Adv Mater 21 (2009) 3410-8.

[24] F. Ajalloueian, S. Zeiai, M. Fossum, J.G. Hilborn, Constructs of electrospun PLGA, compressed collagen and minced urothelium for minimally manipulated autologous bladder tissue expansion, Biomaterials 35 (2014) 5741-8.

[25] W. Sun, D.M. Tiemessen, M. Sloff, R.J. Lammers, E.L. de Mulder, J. Hilborn, B. Gupta, W.F. Feitz, W.F. Daamen, T.H. van Kuppevelt, P.J. Geutjes, E. Oosterwijk, Improving the cell distribution in collagen-coated poly-caprolactone knittings, Tissue Eng Part C Methods 18 (2012) 731-9.

[26] G. Lammers, G. Roth, M. Heck, R. Zengerle, G.S. Tjabringa, E.M. Versteeg, T. Hafmans, R. Wismans, D.P. Reinhardt, E.T. Verwiel, P.L. Zeeuwen, J. Schalkwijk, R. Brock, W.F. Daamen, T.H. van Kuppevelt, Construction of a microstructured collagen membrane mimicking the papillary dermis architecture and guiding keratinocyte morphology and gene expression, Macromol Biosci 12 (2012) 675-91.

[27] S.C. Ling, C.H. Chow, The mechanics of corrugated collagen fibrils in arteries, J Biomech 10 (1977) 71-7.

[28] D.W. Urry, T.M. Parker, Mechanics of elastin: molecular mechanism of biological elasticity and its relationship to contraction, J Muscle Res Cell Motil 23 (2002) 543-59.

[29] D.W. Urry, T. Hugel, M. Seitz, H.E. Gaub, L. Sheiba, J. Dea, J. Xu, T. Parker, Elastin: a representative ideal protein elastomer, Philos Trans R Soc Lond B Biol Sci 357 (2002) 169-84.

[30] L. Debelle, A.M. Tamburro, Elastin: molecular description and function, Int J Biochem Cell Biol 31 (1999) 261-72.

[31] W.F. Daamen, S.T. Nillesen, R.G. Wismans, D.P. Reinhardt, T. Hafmans, J.H. Veerkamp, T.H. van Kuppevelt, A biomaterial composed of collagen and solubilized elastin enhances angiogenesis and elastic fiber formation without calcification, Tissue Eng Part A 14 (2008) 349-60.

[32] L. Cen, W. Liu, L. Cui, W. Zhang, Y. Cao, Collagen tissue engineering: development of novel biomaterials and applications, Pediatr Res 63 (2008) 492-6.

[33] S. Chattopadhyay, R.T. Raines, Review collagen-based biomaterials for wound healing, Biopolymers 101 (2014) 821-33.

[34] R.R. Lareu, I. Arsianti, H.K. Subramhanya, P. Yanxian, M. Raghunath, In vitro enhancement of collagen matrix formation and crosslinking for applications in tissue engineering: a preliminary study, Tissue Eng 13 (2007) 385-91.

[35] J.D. Malcor, D. Bax, S.W. Hamaia, N. Davidenko, S.M. Best, R.E. Cameron, R.W. Farndale, D. Bihan, The synthesis and coupling of photoreactive collagen-based peptides to restore integrin reactivity to an inert substrate, chemically-crosslinked collagen, Biomaterials 85 (2016) 65-77.

[36] M. Barczyk, S. Carracedo, D. Gullberg, Integrins, Cell and Tissue Research 339 (2009) 269-280.

[37] C.N. Grover, J.H. Gwynne, N. Pugh, S. Hamaia, R.W. Farndale, S.M. Best, R.E. Cameron, Crosslinking and composition influence the surface properties, mechanical stiffness and cell reactivity of collagen-based films, Acta Biomater 8 (2012) 3080-90.

[38] K.M. Brouwer, W.F. Daamen, H.R. Hoogenkamp, P.J. Geutjes, I. de Blaauw, W. Janssen-Kessels, W. de Boode, E. Versteeg, R.M. Wijnen, W.F. Feitz, M. Wijnen, T.H. van Kuppevelt, Collagen-Vicryl scaffolds for reconstruction of the diaphragm in a large animal model, J Biomed Mater Res B Appl Biomater 102 (2014) 756-63.

[39] P. Geutjes, L. Roelofs, H. Hoogenkamp, M. Walraven, B. Kortmann, R. de Gier, F. Farag, D. Tiemessen, M. Sloff, E. Oosterwijk, T. van Kuppevelt, W. Daamen, W. Feitz, Tissue engineered tubular construct for urinary diversion in a preclinical porcine model, J Urol 188 (2012) 653-60.

[40] J.E. Nuininga, M.J. Koens, D.M. Tiemessen, E. Oosterwijk, W.F. Daamen, P.J. Geutjes, T.H. van Kuppevelt, W.F. Feitz, Urethral reconstruction of critical defects in rabbits using molecularly defined tubular type I collagen biomatrices: key issues in growth factor addition, Tissue Eng Part A 16 (2010) 3319-28.

[41] N.A. Hosper, A.J. Eggink, L.A. Roelofs, R.M. Wijnen, M.J. van Luyn, R.A. Bank, M.C. Harmsen, P.J. Geutjes, W.F. Daamen, T.H. van Kuppevelt, D.M. Tiemessen, E. Oosterwijk, J.J. Crevels, W.A. Blokx, F.K. Lotgering, P.P. van den Berg, W.F. Feitz, Intra-uterine tissue engineering of full-thickness skin defects in a fetal sheep model, Biomaterials 31 (2010) 3910-9.

[42] N. Annabi, S.M. Mithieux, G. Camci-Unal, M.R. Dokmeci, A.S. Weiss, A. Khademhosseini, Elastomeric Recombinant Protein-based Biomaterials, Biochem Eng J 77 (2013) 110-118.

[43] B. Xu, Y. Li, X. Fang, G.A. Thouas, W.D. Cook, D.F. Newgreen, Q. Chen, Mechanically tissue-like elastomeric polymers and their potential as a vehicle to deliver functional cardiomyocytes, J Mech Behav Biomed Mater 28 (2013) 354-65.

[44] G. Totonelli, P. Maghsoudlou, M. Garriboli, J. Riegler, G. Orlando, A.J. Burns, N.J. Sebire, V.V. Smith, J.M. Fishman, M. Ghionzoli, M. Turmaine, M.A. Birchall, A. Atala, S. Soker, M.F. Lythgoe, A. Seifalian, A. Pierro, S. Eaton, P. De Coppi, A rat decellularized small bowel scaffold that preserves villus-crypt architecture for intestinal regeneration, Biomaterials 33 (2012) 3401-10.

[45] P.D. Verhaegen, H.J. Schouten, W. Tigchelaar-Gutter, J. van Marle, C.J. van Noorden, E. Middelkoop, P.P. van Zuijlen, Adaptation of the dermal collagen structure of human skin and scar tissue in response to stretch: an experimental study, Wound Repair Regen 20 (2012) 658-66.

[46] S. Korossis, F. Bolland, E. Ingham, J. Fisher, J. Kearney, J. Southgate, Review: tissue engineering of the urinary bladder: considering structure-function relationships and the role of mechanotransduction, Tissue Eng 12 (2006) 635-44.

Appendix

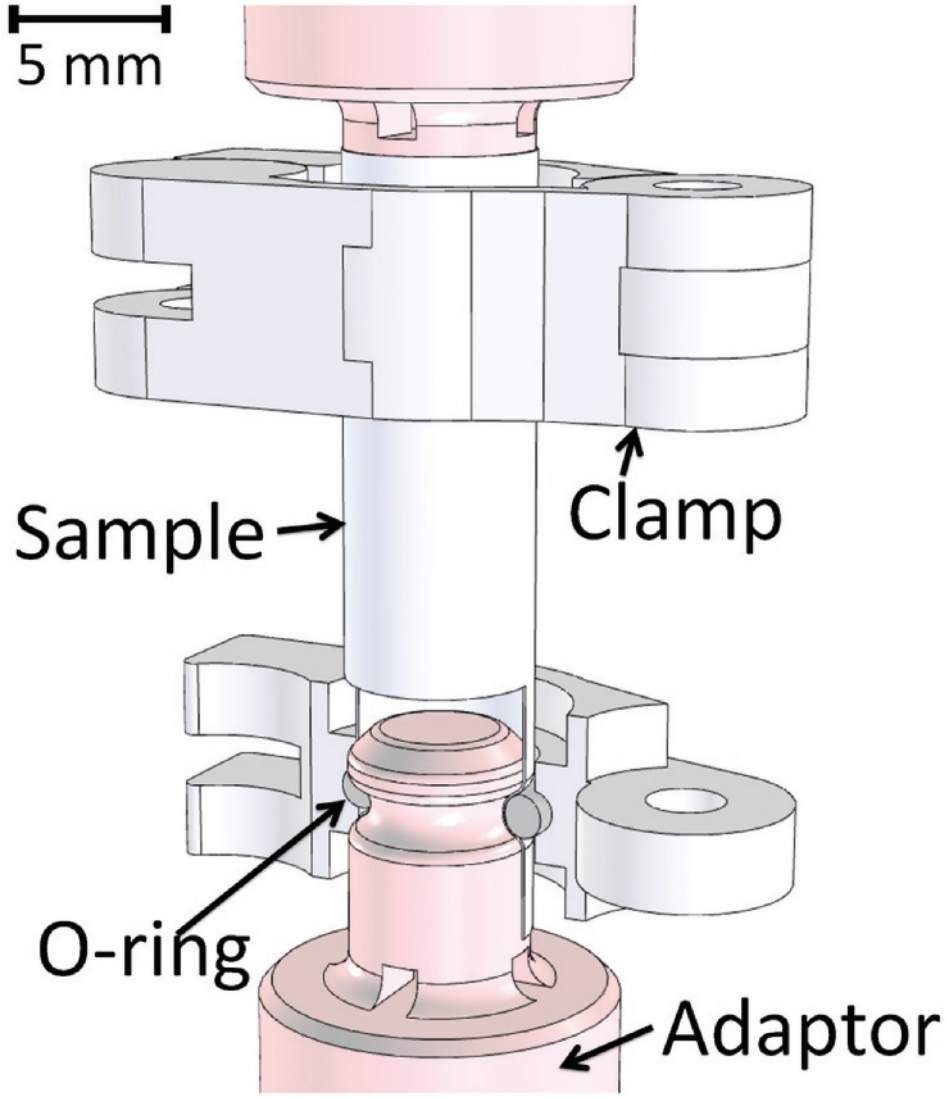


Figure A.1. Schematic overview of custom-made clamp for tubular scaffolds. To allow for the mechanical evaluation of the scaffolds in tubular form a custom-made clamp was made to fit the Zwick/Roell device. The adaptor cylindrical shape could be inserted into a tubular scaffold after which a clamp fitted with a rubber ring could be used to secure the sample in place. Several openings were added to the design of the adaptor to allow for free flow of air/fluid during repeated stretch and relaxation cycles.

TUBULAR COLLAGEN SCAFFOLDS WITH RADIAL ELASTICITY BY SHAPE RECOVERY FOR HOLLOW ORGAN REGENERATION

Luuk R. Versteegden
Kenny A. van Kampen
Heinz P. Janke
Dorien M. Tiemessen
Henk R. Hoogenkamp
Theo G. Hafmans
Edwin A. Roozen
Roger M. Lomme
Harry van Goor
Egbert van Oosterwijk
Wout F. Feitz
Toin H. van Kuppevelt
Willeke F. Daamen

Acta Biomaterialia, APR 2017, volume 52, p1-8.

Abstract

Tubular collagen scaffolds have been used for the repair of damaged hollow organs in regenerative medicine, but they generally lack the ability to reversibly expand in radial direction, a physiological characteristic seen in many native tubular organs. In this study, tubular collagen scaffolds were prepared that display a shape recovery effect and therefore exhibit radial elasticity. Scaffolds were constructed by compression of fibrillar collagen around a star-shaped mandrel, mimicking folds in a lumen, a typical characteristic of empty tubular hollow organs, such as ureter or urethra. Shape recovery effect was introduced by *in situ* fixation using a star-shaped mandrel, 3D-printed clamps and cytocompatible carbodiimide crosslinking. Prepared scaffolds expanded upon increase of luminal pressure and closed to the star-shaped conformation after removal of pressure. In this study, we applied this method to construct a scaffold mimicking the dynamics of human urethra. Radial expansion and closure of the scaffold could be iteratively performed for at least 1000 cycles, burst pressure being 132 ± 22 mmHg. Scaffolds were seeded with human epithelial cells and cultured in a bioreactor under dynamic conditions mimicking urination (pulse flow of 21 s every 2 h). Cells adhered and formed a closed luminal layer that resisted flow conditions.

In conclusion, a new type of a tubular collagen scaffold has been constructed with radial elastic-like characteristics based on the shape of the scaffold, and enabling the scaffold to reversibly expand upon increase in luminal pressure. These scaffolds may be useful for regenerative medicine of tubular organs.

1. Introduction

Regenerative medicine (RM) of tubular or hollow organs is a developing field integrating a number of disciplines including material science, engineering, biomedical and clinical research [1]. Reconstruction of organs such as blood vessels [2], the gastrointestinal tract [3, 4], urogenital [5-7] and respiratory system [8] using RM constructs has been investigated and some constructs, e.g. tracheal scaffolds, have been successfully implanted in patients [9]. The majority of RM approaches apply scaffolds that function as a temporal extracellular matrix to direct tissue generation. Collagen has been extensively used as scaffolding biomaterial for tubular organs, due its wide availability, excellent biocompatibility and straightforward processing methods [10, 11]. However, collagen scaffolds often have limited strength [12] and lack inherent flexibility/elasticity, which restrict their use as tubular scaffolds. Elasticity is an important characteristic for tubular organs, e.g. for those organs such as the esophagus, stomach, ureter and urethra that are closed in the resting state, but expand when food or urine passes [13, 14].

The lumen of the ureter or urethra, for example, is partly star-shaped and closed when no urine is present, but opens during voiding due to urine pressure (Figure 1) [15-17]. After voiding, the smooth muscle layer surrounding the urethral lumen contracts and the lumen returns to its collapsed position by folding of the urethral wall. Upon increase in pressure, unfolding allows expansion of the lumen of the urethra. A rigid collagen scaffold would not comply with the native dynamics of the urethra, and

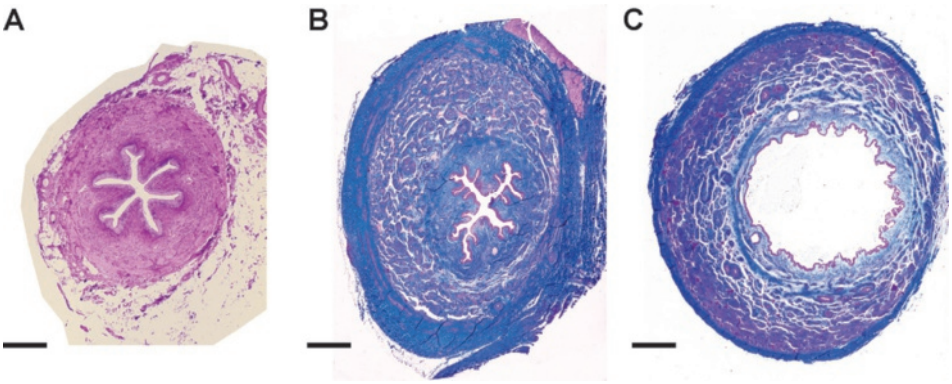


Figure 1. Histological images of cross-sections of the ureter and urethra. A) H&E staining of human ureter showing the star-shaped lumen. B-C) Azan staining of human urethra (corpus spongiosum of the penis) in closed B) and opened C) position showing the characteristic folded lumen that can expand to a circular lumen during urination. By courtesy of Dr. L.G. Poels and Dr. P.H.K. Jap (www.POJA-Collection-Microscopic-Anatomy.com). Scale bars represent 500 µm.

therefore scaffolds should be endowed with elasticity and shape memory. Recently, we developed a method to induce longitudinal elasticity in tubular collagen scaffolds by a process combining compression, corrugation and chemical crosslinking [18]. However, in the human body most tubular structures are fairly rigid in the longitudinal direction and display elasticity in a radial direction. We therefore adapted the methodology to construct tubular scaffolds with radial elasticity in order to comply with the dynamic characteristics of tubular organs. Using this method, the elasticity results from the inherent material properties as well as the unique design of the folded scaffold wall. To illustrate this innovative technology we describe here the construction a star-shaped folded collagen scaffold which exhibits shape recovery effect and is elastic in nature along the radial direction.

2. Materials and methods

2.1. Scaffold construction

Insoluble type I collagen was purified from bovine achilles tendon [19]. A 1.0% (w/v) collagen suspension was prepared by mixing insoluble type I collagen with 0.25 M acetic acid. After overnight swelling, the suspension was homogenized at 2,500 rpm using a laboratory mixer (Silverson L5M-A, Chesham, UK). After removal of air bubbles by centrifugation at 100 g, the suspension was placed inside a 60 ml polypropylene mold (inner diameter 25 mm) with a centered stainless steel mandrel (diameter 15 mm), frozen at -20°C, and lyophilized (Zirbus sublimator 500II, Bad Grund, Germany).

After lyophilization, the wall of the scaffold was compressed by placing the scaffold including mandrel between two flat aluminum objects and applying pressure and a rolling motion until a thin tubular sheet of collagen was obtained (Figure 2A). The mandrel was removed from the scaffold and a custom star-shaped mandrel (Figure 2B) printed from polyamide 12 (Nylon-12, Stratasys, Eden Prairie, Minnesota, USA) using a Fortus 360mc 3D-printer (Stratasys) was inserted into the lumen of the compressed scaffold. The scaffold was compressed around this five tip star-shaped mandrel. To fixate the scaffold, star-shaped 3-D printed compression clamps (Figure 2B) were placed around the scaffold, followed by crosslinking at ambient temperature for 3 h using 33 mM N-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, Merck Schuchardt OHG, Hohenbrunn, Germany) and 6 mM N-hydroxysuccinimide (NHS, Fluka Chemie AG, Buchs, Switserland) in 50 mM 2-morpholineoethane sulphonic acid (MES buffer, pH 5.0) (USB, Ohio, USA) containing 40% ethanol [19]. The constructs

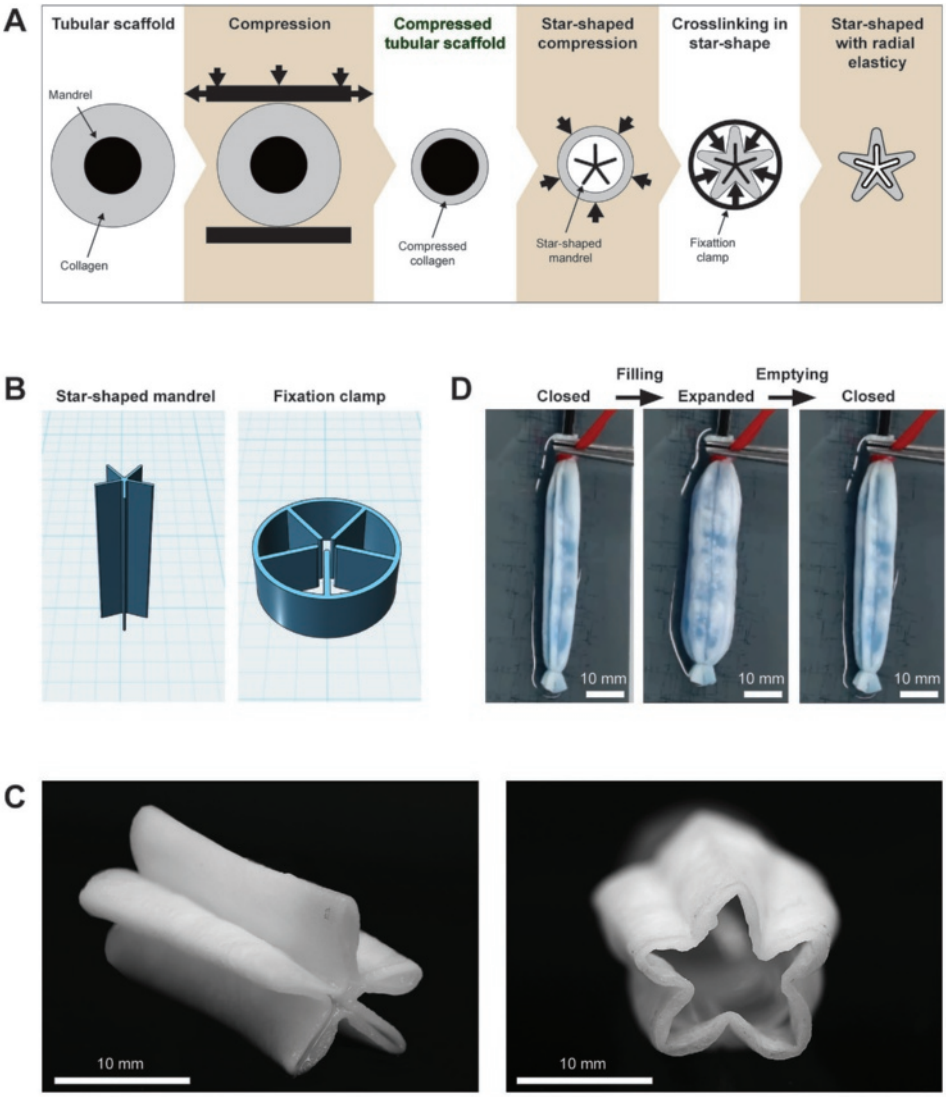


Figure 2. Construction of a folded, star-shaped scaffold with radial elasticity. A) Schematic representation of construction process. A porous tubular collagen scaffold is compressed between two surfaces under a rolling motion resulting in a compressed tubular scaffold. This scaffold is manually compressed around a five point star-shaped mandrel, fixed with custom-made clamps and crosslinked in the star-shape position using EDC/NHS. B) 3-D printed polyamide mandrel and clamp used in production process. C) Macroscopic view of scaffold in closed and partially open position. D) Expansion and relaxation (folding, unfolding) of scaffold after injection and removal of water (with bromophenol blue for detection of potential leakage).

were incubated for 3 h at ambient temperature, but clamps were removed after 2 h. After crosslinking, the scaffolds were washed with 0.1 M Na_2HPO_4 (Merck, Darmstadt, Germany), 1.0 M NaCl, 2.0 M NaCl and demineralized water. Constructs were stored in 70% ethanol at ambient temperature until further use.

2.2. Scaffold characterization

2.2.1. Macroscopic characterization

Macroscopic images were photographed using a Canon EOS-1DX Mark II with Canon macro objective (Canon, Melville, NY, USA). Supplementary movies were recorded with a Sony Cybershot DSC-H10.

2.2.2. Scanning Electron Microscopy

Scanning electron microscopy (SEM) was used to analyze the morphology and structure of the scaffolds. Scaffolds were washed with demineralized water, frozen at -80°C and lyophilized. The dry samples were placed on a stub with double-sided carbon tape and sputtered with an ultrathin gold layer for 60 s (Scancoat Six Sputter Coater, Edwards, Crawley, United Kingdom). Examination was performed in a Sigma300 scanning electron microscope (Carl Zeiss AG, Oberkochen, Germany) at an accelerating voltage of 10 kV.

2.2.3. Mechanical characterization

Mechanical properties of the scaffolds were characterized using fatigue and burst pressure analysis using a Zwick/Roell Z2.5 tensile testing apparatus (Zwick/Roell, Ulm, Germany). Using a 60 ml syringe, water was injected into the star-shaped scaffold, which was closed at one end with a clamp and at the other end with a tube connected to a pressure transducer (Edwards, Irvine California USA) connected to the tensile tester software. Bromophenol blue was added to the water to detect leakage through the scaffold. For burst pressure analysis (n=8), the scaffold was filled at a velocity of 13 ml/min until rupture. To analyze fatigue characteristics (n=8), the scaffold was filled with 10 ml water in 5 s and emptied in 10 s. This cycle was repeated 1000 times and for every cycle the peak pressure was measured. After the fatigue test, scaffolds were subjected to a burst pressure analysis. The burst pressure of a scaffold with a round lumen (compressed tubular scaffold in Figure 2A), not subjected to star-shaped compression, was also analyzed to investigate its effect on radial strength. To compare the mechanical properties of the scaffold with native tissue, the burst pressure of excised pig urethras (n=5) was analyzed similarly.

2.3. Culture of urothelial cells on scaffolds using a bioreactor system and voiding conditions

2.3.1. Cell culture

SCaBER cells (ATCC, Manassas, VA, USA), a urothelial cell line derived from a squamous bladder carcinoma, were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium with Glutamax™ (GIBCO, Life Technologies) enriched with 10% fetal bovine serum (GE healthcare, PAA Laboratories, Pasching, Austria), 100 IU/ml penicillin and 100 µg/ml streptomycin (GIBCO, Life Technologies) at 37°C and 5% CO_2. At 80% confluency, cells were passaged using 0.05% (w/v) trypsin-EDTA (Life Technologies). SCaBER cells were used as we were merely interested in cellular attachment and maintenance. SCaBER cells can be cultured in large quantities needed for the bioreactor experiment and have previously been used as a first *in vitro* evaluation step for biomaterials [20].

2.3.2. Cell seeding on star-shaped scaffold

Scaffolds (7 cm length, 15 mm inner diameter) were extensively washed in 0.1 M phosphate buffered saline (PBS, pH 7.4) (4x 1 h and 1x overnight using at least 250 mL) and subsequently sterilized by 25 kGy gamma irradiation (Synergy Health, Ede, The Netherlands). The sterile star-shaped scaffolds were closed at both ends by 5-0 Monocryl™ (Johnson & Johnson) sutures to prevent cells from leaking out. Next, 8.0 x 10^6 cells in 7 ml were injected into the lumen of the scaffold via a 25G Microlance™ (BD, Drogheda, Ireland) needle. Seeded scaffolds were transferred to a 50 ml tube with 25 ml culture medium and rotated at 10 rpm in a 37°C incubator. After 24 h, sutures were removed and scaffolds were transferred to a T75 flask (Greiner Bio-One) and cultured for 3 days at 37°C and 5% CO_2.

2.3.3.Dynamic cell culture in bioreactor

After 3 days of static culture, one quarter of the scaffold was transferred to a culture flask for further static culture as control. The other part was transferred to a Bose ElectroForce bioreactor (Eden Prairie, Minnesota, USA) using a custom-made reaction chamber (Lifetec Group, Eindhoven, The Netherlands). Tubes were connected to both ends of the star-shaped scaffold and the scaffolds were subjected to pulse flow of 21 s every 2 h using a gear pump (Bose, Eden Prairie, Minnesota, USA), mimicking the average voiding characteristics in men [21]. The bioreactor contained 125 ml culture medium and was connected to a reservoir containing 250 ml culture medium to prevent depletion of culture medium. Cell-seeded scaffolds were cultured for 3 days in the bioreactor system. Three independent experiments were performed.

2.3.4. Morphological evaluation

Sample preparation for scanning electron microscopy

Part of the cultured scaffold was fixed with 2% glutaraldehyde in 0.1 M phosphate buffer (PB, pH 7.4), followed by three washings with PB. After dehydration with an ascending gradient of ethanol solutions (30, 50, 70 and 100% v/v), samples were dried using a Polaron Critical Point Drier (Quorum Technologies, Ringmer, United Kingdom). Dry samples were analyzed with SEM as described in section 2.2.2.

(Immuno)histochemistry

Scaffolds were harvested and fixed in 4% (w/v) paraformaldehyde in phosphate buffered saline (PBS, pH 7.4). After washing with 0.1 M PBS, an ascending series of ethanol (70, 96, 99, 100% v/v) and xylene, the scaffolds were embedded in paraffin and sectioned (5 µm) using a Microm HM340E microtome (ThermoFischer). Slides were deparaffinized and stained with haematoxylin and eosin (HE).

In addition, scaffolds were embedded in TissueTek (Sakura Finetek Europe, Alphen aan de Rijn, The Netherlands), frozen using dry-ice cooled isopentane (2-methylbutane, Sigma Aldrich, St. Louis, USA) and ultrathin sections (5 µm) were obtained using a Microm HM 500OM Cryostat Microtome (Heidelberg Instrument Mikrotecknik, Heidelberg, Germany). Slides were immunostained for type I collagen, cytokeratin 18, and cell nuclei (4',6-diamidino-2-phenylindole, DAPI). Slides were blocked with 5% (v/v) normal goat serum (Sigma Aldrich, St. Louis MO, USA) in PBS with 1% (v/v) bovine serum albumin (BSA, Merck, Darmstadt, Germany) for 15 min. Slides were incubated for 30 min with a mouse-anti-human cytokeratin 18 antibody (1:400, Mubio Products BV, Maastricht, The Netherlands) followed an incubation of 30 min with a rabbit-anti-bovine type I collagen antibody (1:250, Millipore, Cambridge, UK) followed by 3 washings with PBS. Secondary antibodies Alexa fluor 594-conjugated goat-anti-mouse IgG antibody and Alexa fluor 488-conjugated goat-anti-rabbit (both Invitrogen, Eugene, OR, USA) were consecutively incubated for 30 min followed by 3 washings with PBS. Cell nuclei were stained with 5 µg/ml DAPI (Sigma Aldrich, St. Louis MO, USA) for 15 min. Sections were washed 3 times with PBS and mounted with coverslips using Dako Fluorescence Mounting Medium (Agilent Technologies, Amstelveen, The Netherlands). Slides were analyzed using a Leica DM600B microscope (Leica Microsystems) and images were processed using ImageJ 1.48v (National Institutes of Health, USA). Brightness and contrast were manually adjusted for all photos including controls.

2.4. Statistical analysis

GraphPad PRISM 5, version 5.03 (GraphPad Software Inc, La Jolla, CA, USA) was used for statistical analysis. For the burst pressure tests (section 2.2.3), different groups were compared using a one-way ANOVA with Bonferroni post-hoc tests.

3. Results

3.1. Morphological characterization

Tubular collagen scaffolds were created by swelling, homogenizing, freezing and freeze-drying of a fibrillar type I collagen suspension. To mimic the folded wall morphology of an empty urethra (Figure 1), compression around a five tip star-shaped mandrel in combination with carbodiimide crosslinking was applied which resulted in a scaffold with a star-shaped lumen which was almost closed (Figure 2). The lumen could be expanded to the unfolded state with a diameter similar to the one before the star-shaped compression (15 mm) by applying mechanical pressure (Figure 2D). When the pressure was removed, the lumen automatically returned to the star-shape in which it was crosslinked (shape memory, see movie 1). The shape-memory mechanism is entropy driven and observed in aqueous, but not dry, conditions (see movie 2).

SEM analysis of cross-sections of the scaffolds showed a star-shaped lumen and a porous inner structure of the luminal region (Figure 3A,B) and the tip region (Figure 3C,D). Further analyses revealed that the luminal surface of the scaffold was predominantly closed and smooth (Figure 3E,F), whereas the outside was also closed but had a more rough surface (result not shown). The thickness of the wall varied from approximately 150 µm (most central part of the fold) to 400 µm (most distal part of the fold), in line with the preparation method in which the walls are manually compressed against the mandrel.

3.2. Mechanical characterization

To analyze biomechanical properties, the scaffolds were subjected to burst pressure and fatigue tests. The star-shaped scaffolds had an average burst pressure of 132 ± 22 mmHg (n=8) (Figure 4A). Scaffolds that were first exposed to 1000 stretch-relax cycles (fatigue test) followed by burst pressure analysis showed a slightly lower, non-significant, burst pressure of 111 ± 14 mmHg (n=8). Round scaffolds that were not subjected to star-shaped compression (compressed tubular scaffold from Figure 2A) had an average burst pressure of 52 ± 21 mmHg, indicating that the star-shaped

compression added radial strength to the scaffolds. Excised native pig urethras with part of the muscular tissue still present showed a burst pressure of 274 ± 93 mmHg (n=5). The fatigue test further indicated that the scaffolds kept their mechanical characteristics after exposure to 1000 filling and emptying cycles. The peak pressure measured during the filling cycles slightly decreased over time, especially in the initial phase, but stabilized in later cycles. In Figure 4B a representative graph of the peak pressure for every cycle is given showing a decrease in pressure from approximately 28 mmHg to 24 mmHg.

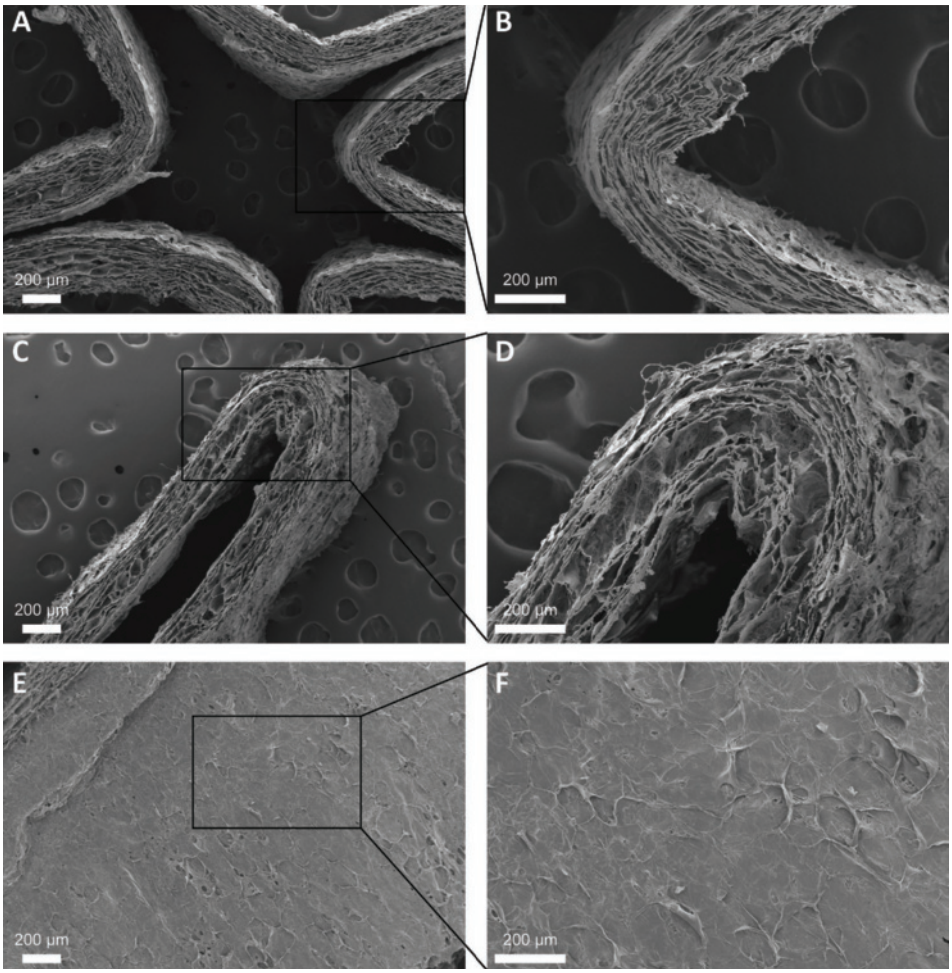


Figure 3. Microscopic evaluation of the star-shaped collagen scaffold. A-D) Cross-sectional SEM images of the overall luminal region (A+B) with the tip region (C+D) of the star-shaped scaffold showing the general morphology and porous structure. E+F) SEM images of the luminal surface of the scaffold showing a mainly smooth and closed surface without pores.

3.3. Dynamic cell culture in bioreactor

To investigate whether cells remain attached to the star-shaped scaffold under dynamic conditions that they would experience under voiding conditions *in vivo,* SCaBER cells were seeded on scaffolds and cultured in a bioreactor system. Cells were first cultured for 3 days under static conditions to allow adherence to the scaffold. After this initial culture phase, the lumen of the scaffold was partly covered with monolayers of SCaBER cells (see Appendix, Figure A.1).

Next, the cell-seeded scaffolds were cultured for 3 more days in a bioreactor under dynamic flow conditions reflecting human voiding behavior. All scaffolds that were dynamically stimulated showed patency without graft failure. Shape memory was observed during the whole stimulation process. For comparison, static culture conditions were also applied. H&E staining of cross-sections of the scaffold showed that cells were distributed throughout the entire scaffold and formed multilayers under dynamic conditions (Figure 5A).

The thickness of the cell layers varied from a monolayer up to a layer of 5 to 6 cells in thickness (Figure 5A, inset), with about 75% of the lumen covered (Figure 5B). Cells

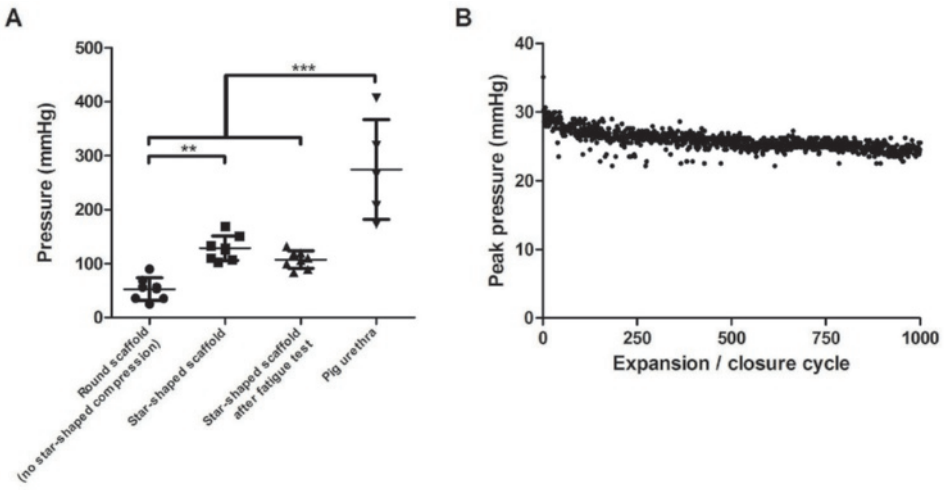


Figure 4. Mechanical properties of the tubular star-shaped, elastic scaffold. A) Burst-pressure of round scaffolds without star-shaped compression, star-shaped scaffolds before and after fatigue test (both n=8), and of native pig urethras (n=5). Tubes were closed at both ends and water was pumped into the lumen until rupture while continuously monitoring the pressure. Bars represent mean ± standard deviation. One way ANOVA with Bonferroni post-hoc test, *** P<0.0001. B) Representative graph of a fatigue test of 1000 expansion and closure cycles. Each dot represents a peak pressure at one specific cycle.

resided on the scaffold's luminal surface with almost no penetration into the scaffold wall.

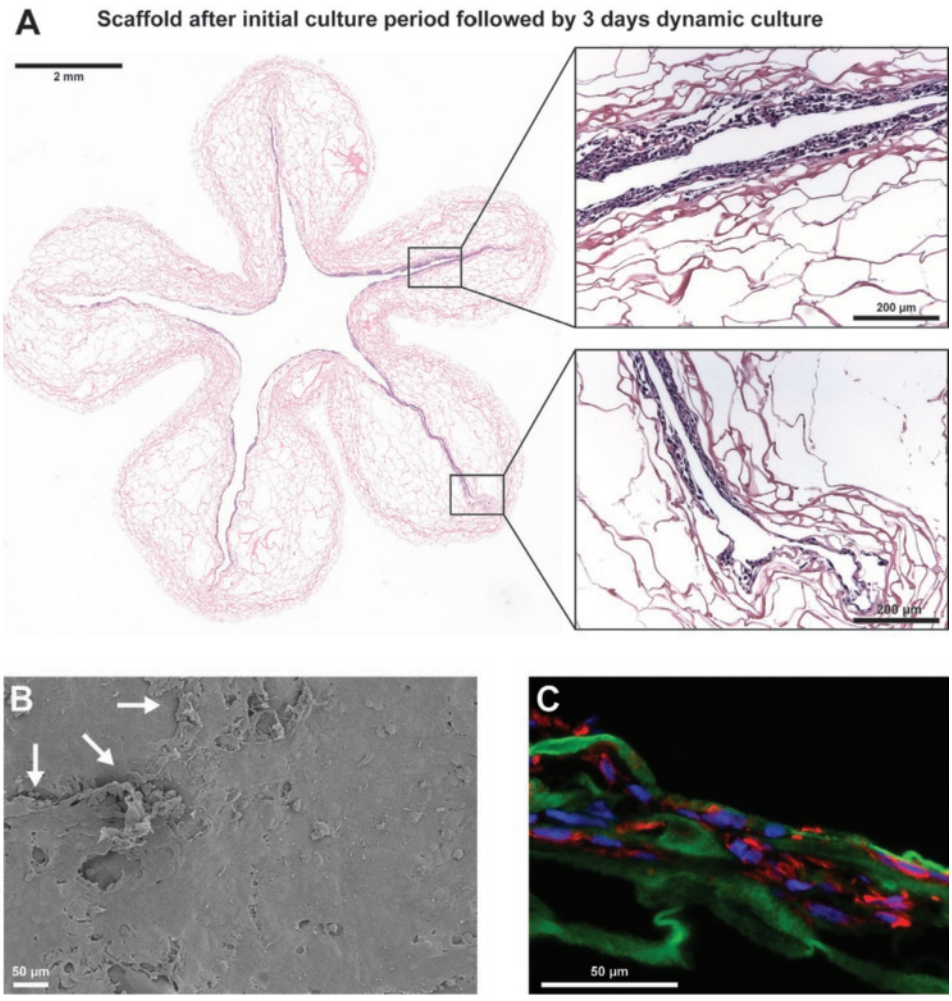


Figure 5. Star-shaped scaffold after initial culture period followed by 3 days dynamic culture. A) H&E staining of cross-sections of cell-seeded star-shaped scaffolds. Panoramic view of H&E stained scaffolds cultured with cells under dynamic conditions for 3 days showing almost entire coverage of the luminal surface. Enlargements show that cells did not penetrate the scaffold but remained at the luminal surface. B) SEM image of the luminal surface of a scaffold cultured in a bioreactor showing cells partly covering the luminal surfaces. White arrows indicate the edge of the cell sheet. C) Immunostaining of dynamically cultured scaffold stained for cell nuclei with DAPI (blue), type I collagen (green) and cytokeratin 18 (red) showing that cells mainly reside on the scaffold's surface and express cytokeratin.

The mechanical stress caused by the pulse flow of 21 s every 2 h did not cause detachment of the cells from the scaffold when compared to the statically cultured scaffold (see Appendix A, Figure A.2.). Immunostaining for cytokeratin 18 indicated the epithelial character of the cultured cells (Figure 5C).

In general, no differences were observed between scaffolds cultured under static or dynamic conditions, indicating that the pulse flow did not have an effect on cellular attachment and maintenance. Moreover, cell numbers increased in time for both the static and dynamic condition as assessed by H&E staining (see Figure 5 and Appendix Figure A.1+A.2), indicating cytocompatibility.

4. Discussion

Tubular collagen scaffolds have been used in regenerative medicine of hollow organs such as blood vessels [2, 22], urogenital system [6, 23] and gastro-intestinal tract [24], but clinical translation is still limited. A number of methods have been developed to improve the morphology, 3D pore architecture and fiber alignment to enhance tissue regeneration [25]. To improve strength and to control biodegradability, crosslinking is often applied. In addition, biological active components such as growth factors [26] and glycosaminoglycans [19] have been used to stimulate the regeneration process. However, less attention has been paid to physiologically crucial mechanical properties such as elasticity and folding-unfolding. Especially for the many dynamic organs in the body ranging from lung to skin and from blood vessels to urethra, scaffolds should be developed with biomechanical characteristics that comply with the native organ, thus facilitating regeneration. A number of organs in the body physiologically “collapse” and become flat-folded when no food (e.g. esophagus) or urine (e.g. urethra) is present. In an empty state these organs are characterized by the process of infolding, in which the wall folds upon itself and forms a folded, star-shaped, lumen. Folding is an intrinsic characteristic of the wall, primarily caused by muscle tissue and the appropriate extracellular matrix. Mimicking these characteristics may be an advantage for regenerative medicine of such organs. In this study we aimed to construct tubular scaffolds with radial elastic-like characteristics enabling the scaffold to expand and close in radial orientation, using only fibrillar type I collagen as a biomaterial.

As a proof of concept a star-shaped, folded scaffold was prepared to mimic the morphology and dynamics of the urethra. The technology used, based on inducing

entropy-driven elasticity by a molding and crosslinking process, is easily adaptable to other organs by modification of the molds and mandrels used. The scaffold presented here may be further optimized to have a diameter similar to the average diameter of the male urethra; approximately 6 mm in its expanded position during voiding [27]. Patient-specific adaptation of e.g. size and diameter can easily be realized by adapting the molds and mandrels.

The burst pressure of the scaffolds prepared was 132 ± 22 mmHg. A recent study of Pinnagoda *et al.* showed that a burst pressure strength of approximately 77.4 ± 2.4 cmH_2O, comparable to 56.9 ± 1.8 mmHg, would be sufficient for successful repair of the urethra in a rabbit model [11]. For females, the mean urethral closure pressure is 60 mmHg in standing position [28]. For males the maximal urethral closure pressure ranges up to 150 cmH_2O (110 mmHg) [29]. Vardar *et al.* showed that collagen scaffolds for ureter repair with a maximum intra-luminal pressure of 22.4 ± 0.1 cmH_2O (16.5 ± 0.1 mmHg) were in range with physiological pressures in adults [30]. Overall, the developed star-shaped scaffold is expected to be strong enough to withstand intra-luminal and physiological relevant pressures for urethra and ureter repair, but more dedicated experiments should be performed to confirm this.

Collagen scaffolds crosslinked with EDC and NHS are generally biocompatible *in vivo* [31, 32] and cytocompatible *in vitro* [10, 19], also in combination with compression techniques [18]. With our strategy to implant acellular scaffolds, cells from adjacent tissue migrating into the scaffold will experience mechanical stress when the scaffold expands and closes. We therefore assessed cellular behavior under dynamic culture conditions in a bioreactor mimicking the mechanical stress to which residing urothelial cells are exposed in the urethra (pulse flow of 21 s every 2 h).

Seeded SCaBER cells did not detach from the scaffold under static and dynamic culture conditions. About 75% of the lumen was covered by one or more layers of cells after three days of dynamic culturing, an important aspect as the urothelium functions as barrier to prevent leakage of urine to the underlying tissue [33]. Longer culturing may result in complete coverage. Overall, the expansion and closure of the scaffold does not seem to affect cytocompatibility. Our strategy for *in vivo* implantation is the use of acellular constructs without prior cell seeding where endogenous urothelial cells will line the lumen of the scaffold. This approach is feasible as indicated by Nuininga *et al.* [34].

To the best of our knowledge, collagen scaffolds with radial elastic-like characteristics have not been reported before. This scaffold type may broaden the opportunities for use of collagen to regenerate hollow organs. By varying the concentration of collagen, the casting molds, and the extent of crosslinking, tailor-made tubular constructs can be prepared that have the ability to expand and automatically close. By using this technique, scaffolds can be prepared that comply with movements of dynamic tissues, such as those present in the urogenital and gastrointestinal system.

5. Conclusion

In this paper, an enabling technology was developed to construct tubular collagen scaffold with elastic-like characteristics in a radial direction. Scaffolds expanded upon increase of luminal pressure, and automatically collapsed after the pressure was released (shape recovery). Scaffolds were cytocompatible. The technology may be useful for the construction of scaffolds for the regeneration of tubular tissue with radial elasticity such as blood vessels, ureter, urethra, esophagus, bowel or bile duct.

6. Acknowledgements

Mark van de Hei from the Technical Support Group of the Radboud University Faculty of Social Sciences is acknowledged for help with the design and manufacturing of the custom made mandrels and clamps. Dr. L.G. Poels and Dr. P.H.K. Jap from the Dept. of Anatomy (Radboudumc) were acknowledged for providing images of human urethra and ureter. Corien Oostendorp from the Dept. of Biochemistry (Radboudumc) is acknowledged for her assistance with immunostainings. This study was financially supported by PIDON (NovioTissue project), a combined subsidiary program of the Dutch Ministry of Economic Affairs, and the states of Gelderland and Overijssel (PID101020). The source of funding had no other involvement in this publication.

7. Supplemental information

Supplemental information is available at https://doi.org/10.1016/j.actbio.2017.02.005.

8. References

[1] Hendow EK, Guhmann P, Wright B, Sofokleous P, Parmar N, Day RM. Biomaterials for hollow organ tissue engineering. Fibrogenesis & tissue repair 2016;9:3.

[2] Huang AH, Niklason LE. Engineering of arteries in vitro. Cellular and Molecular Life Sciences 2014;71:2103-18.

[3] Maghsoudlou P, Eaton S, De Coppi P. Tissue engineering of the esophagus. Seminars in pediatric surgery 2014;23:127-34.

[4] Bitar KN, Zakhem E. Bioengineering the gut: future prospects of regenerative medicine. Nature reviews Gastroenterology & hepatology 2016.

[5] Sloff M, Simaioforidis V, de Vries R, Oosterwijk E, Feitz W. Tissue engineering of the bladder--reality or myth? A systematic review. The Journal of urology 2014;192:1035-42.

[6] de Jonge PK, Simaioforidis V, Geutjes PJ, Oosterwijk E, Feitz WF. Recent advances in ureteral tissue engineering. Current urology reports 2015;16:465.

[7] Cui T, Terlecki R, Atala A. Tissue engineering in urethral reconstruction. Archivos espanoles de urologia 2014;67:29-34.

[8] Fishman JM, Wiles K, Lowdell MW, De Coppi P, Elliott MJ, Atala A, Birchall MA. Airway tissue engineering: an update. Expert opinion on biological therapy 2014;14:1477-91.

[9] Hamilton NJ, Kanani M, Roebuck DJ, Hewitt RJ, Cetto R, Culme-Seymour EJ, Toll E, Bates AJ, Comerford AP, McLaren CA, Butler CR, Crowley C, McIntyre D, Sebire NJ, Janes SM, O'Callaghan C, Mason C, De Coppi P, Lowdell MW, Elliott MJ, Birchall MA. Tissue-Engineered Tracheal Replacement in a Child: A 4-Year Follow-Up Study. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons 2015;15:2750-7.

[10] Chattopadhyay S, Raines RT. Review collagen-based biomaterials for wound healing. Biopolymers 2014;101:821-33.

[11] Koens MJ, Geutjes PJ, Faraj KA, Hilborn J, Daamen WF, van Kuppevelt TH. Organ-specific tubular and collagen-based composite scaffolds. Tissue engineering Part C, Methods 2011;17:327-35.

[12] Dong C, Lv Y. Application of Collagen Scaffold in Tissue Engineering: Recent Advances and New Perspectives. Polymers 2016;8:42.

[13] Long JD OR. Anatomy, histology, embryology, and developmental abnormalities of the esophagus. In: Feldman M FL, Sleisenger MH, editor. Gastrointestinal and Liver Diseases. 9th ed: Saunders, Elsevier.

[14] Woodburne RT, Lapides J. The ureteral lumen during peristalsis. The American journal of anatomy 1972;133:255-8.

[15] Zecchi-Orlandini S, Gulisano M, Orlandini GE, Holstein AF. Scanning electron microscopic observations on the epithelium of the human spongy urethra. Andrologia 1988;20:132-7.

[16] Watanabe H, Takahashi S, Ukimura O. Urethra actively opens from the very beginning of micturition: a new concept of urethral function. International journal of urology : official journal of the Japanese Urological Association 2014;21:208-11.

[17] K.M. Anderson DAH. The pressure flow study. In: A.C. Peterson MOF, editor. Practical Urodynamics for the clinician: Springer; 2016. p. 66-76.

[18] Versteegden LR, Hoogenkamp HR, Lomme RM, van Goor H, Tiemessen DM, Geutjes PJ, Oosterwijk E, Feitz WF, Hafmans TG, Verdonschot N, Daamen WF, van Kuppevelt TH. Design of an elasticized collagen scaffold: A method to induce elasticity in a rigid protein. Acta Biomater 2016.

[19] Pieper JS, Oosterhof A, Dijkstra PJ, Veerkamp JH, van Kuppevelt TH. Preparation and characterization of porous crosslinked collagenous matrices containing bioavailable chondroitin sulphate. Biomaterials 1999;20:847-58.

[20] Sun W, Tiemessen DM, Sloff M, Lammers RJ, de Mulder EL, Hilborn J, Gupta B, Feitz WF, Daamen WF, van Kuppevelt TH, Geutjes PJ, Oosterwijk E. Improving the cell distribution in collagen-coated poly-caprolactone knittings. Tissue engineering Part C, Methods 2012;18:731-9.

[21] Yang PJ, Pham J, Choo J, Hu DL. Duration of urination does not change with body size. Proceedings of the National Academy of Sciences 2014;111:11932-7.

[22] Buijtenhuijs P, Buttafoco L, Poot AA, Daamen WF, van Kuppevelt TH, Dijkstra PJ, de Vos RA, Sterk LM, Geelkerken BR, Feijen J, Vermes I. Tissue engineering of blood vessels: characterization of smooth-muscle cells for culturing on collagen-and-elastin-based scaffolds. Biotechnology and applied biochemistry 2004;39:141-9.

[23] Nuininga JE, van Moerkerk H, Hanssen A, Hulsbergen CA, Oosterwijk-Wakka J, Oosterwijk E, de Gier RP, Schalken JA, van Kuppevelt TH, Feitz WF. Rabbit urethra replacement with a defined biomatrix or small intestinal submucosa. European urology 2003;44:266-71.

[24] Bitar KN, Raghavan S. Intestinal Tissue Engineering: Current Concepts and Future Vision of Regenerative Medicine in the Gut. Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society 2012;24:7-19.

[25] Hapach LA, VanderBurgh JA, Miller JP, Reinhart-King CA. Manipulation of in vitro collagen matrix architecture for scaffolds of improved physiological relevance. Physical biology 2015;12:061002.

[26] Nillesen STM, Geutjes PJ, Wismans R, Schalkwijk J, Daamen WF, van Kuppevelt TH. Increased angiogenesis and blood vessel maturation in acellular collagen–heparin scaffolds containing both FGF2 and VEGF. Biomaterials 2007;28:1123-31.

[27] A.J. Wein LRK, A.C. Novick, A.W. Partin, C.A. Peters. Cambell-Walsch Urology. 10th ed: Elsevier Saunders; 2012.

[28] Henriksson L, Ulmsten U, Andersson KE. The effect of changes of posture on the urethral closure pressure in healthy women. Scandinavian journal of urology and nephrology 1977;11:201-6.

[29] Kirkeby HJ, Sorensen S, Poulsen EU. Urethral pressure variations in healthy male volunteers. Urological research 1989;17:191-5.

[30] Vardar E, Engelhardt EM, Larsson HM, Mouloungui E, Pinnagoda K, Hubbell JA, Frey P. Tubular Compressed Collagen Scaffolds for Ureteral Tissue Engineering in a Flow Bioreactor System. Tissue engineering Part A 2015;21:2334-45.

[31] Brouwer KM, Daamen WF, Reijnen D, Verstegen RH, Lammers G, Hafmans TG, Wismans RG, van Kuppevelt TH, Wijnen RM. Repair of surgically created diaphragmatic defect in rat with use of a crosslinked porous collagen scaffold. Journal of tissue engineering and regenerative medicine 2013;7:552-61.

[32] Nillesen ST, Geutjes PJ, Wismans R, Schalkwijk J, Daamen WF, van Kuppevelt TH. Increased angiogenesis and blood vessel maturation in acellular collagen-heparin scaffolds containing both FGF2 and VEGF. Biomaterials 2007;28:1123-31.

[33] Romih R, Korošec P, de Mello W, Jezernik K. Differentiation of epithelial cells in the urinary tract. Cell and Tissue Research 2005;320:259-68.

[34] Nuininga JE, Koens MJ, Tiemessen DM, Oosterwijk E, Daamen WF, Geutjes PJ, van Kuppevelt TH, Feitz WF. Urethral reconstruction of critical defects in rabbits using molecularly defined tubular type I collagen biomatrices: key issues in growth factor addition. Tissue engineering Part A 2010;16:3319-28.

Appendix

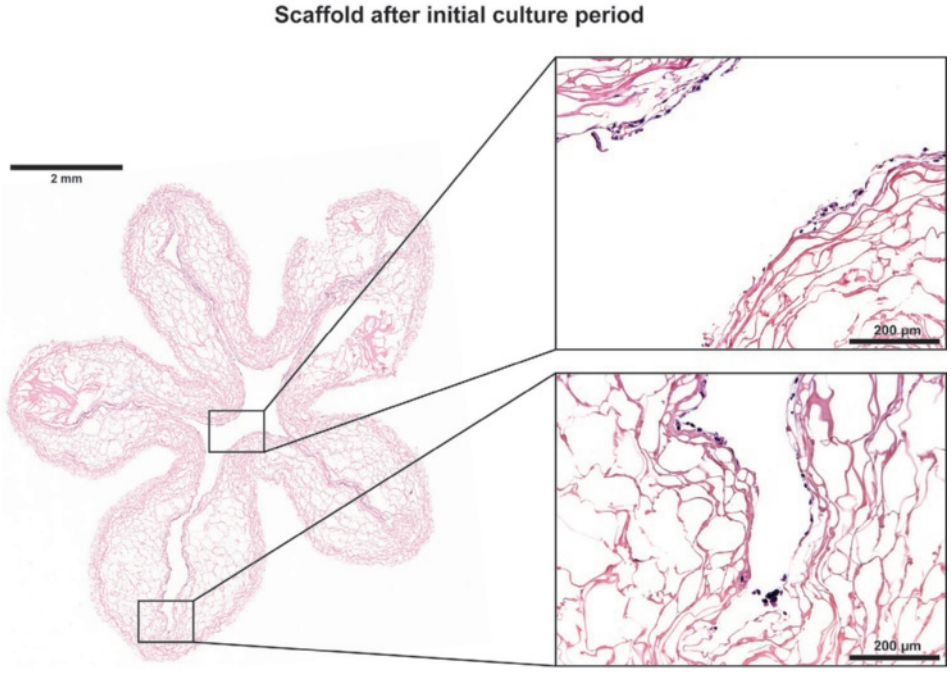


Figure A.1. Star-shaped scaffold after initial (3 days) culture period. Panoramic view of H&E staining of cross-sections of cell-seeded star-shaped scaffold cultured for 3 days showing that cells spread throughout the entire lumen of the scaffold. Enlargements show that the luminal surface is only partly covered with SCaBER cells.

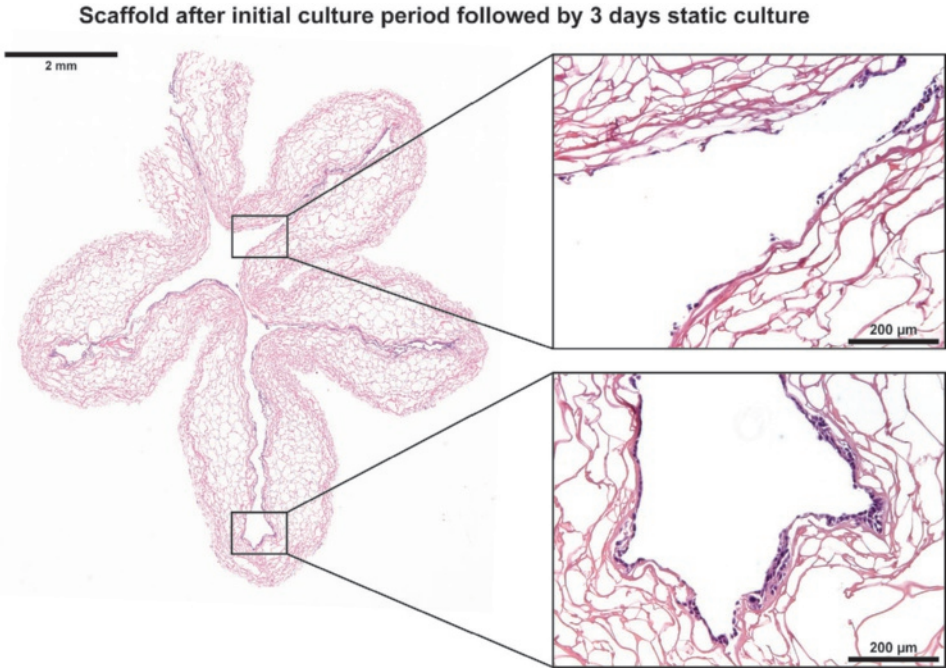


Figure A.2. Star-shaped scaffold after initial culture period followed by 3 days static culture. Panoramic view of H&E staining of cross-sections of cell-seeded star-shaped scaffold cultured under static conditions showing that cells maintained in the scaffold. Enlargements show that especially in the tip region the luminal surface is completely covered with a monolayer of cells, while the surface is only partly covered in the inner region of the lumen.

SELF-EXPANDABLE TUBULAR COLLAGEN IMPLANTS

Luuk R. Versteegden*
Marja ter Meer*
Roger M. Lomme
Daan A. van der Vliet
Leo J. Schultze Kool
Toin H. van Kuppevelt
Willeke F. Daamen
*Contributed equally

Provisionally accepted

Abstract

Collagen has been extensively used as a biomaterial, yet for tubular organ repair synthetic polymers or metals (*e.g.* stents) are typically used. In this study we report a novel type of tubular implant solely consisting of type I collagen, suitable to self-expand in case of minimal invasive implantation. Potential benefits of this collagen scaffold over conventional materials include improved endothelialization, biodegradation over time and possibilities to add bioactive components to the scaffold, such as anti-coagulants.

Implants were prepared by compression of porous scaffolds consisting of fibrillar type I collagen (1.0-2.0% (w/v)). By applying carbodiimide crosslinking to the compressed scaffolds in their opened position, entropy-driven shape memory was induced. The scaffolds were subsequently crimped and dried around a guidewire. Upon exposure to water, crimped scaffolds deployed within 15-60 s (depending on the collagen concentration used), thereby returning to the original opened form. The scaffolds were cytocompatible as assessed by cell culture with human primary vascular endothelial and smooth muscle cells. Compression force required to compress the open scaffolds increased with collagen content from 16 mN to 32 mN for 1.0 to 2.0% (w/v) collagen scaffolds.

In conclusion, we report the first self-expandable tubular implant consisting of solely type I collagen that may have potential as a biological vascular implant.

The main function of tubular structures, such as blood vessels or bile ducts, is the transportation of fluids. These structures may become blocked due to atherosclerotic diseases (e.g. stenoses) or malignancies. Biomaterials have proven to be effective tools to ensure patency of tubular structures. Currently the majority of devices for clinical use are permanent implants, mostly metals or medical textiles such as expanded polytetrafluoroethylene (ePTFE). The concept of biodegradability has gained interest over the last years as it comes with obvious benefits. The absence of foreign body material at long term follow-up may reduce the risk of complications such as occlusion of blood vessels and infection [1]. Currently used biodegradable implants for tubular tissues, often prepared from synthetic polymers or magnesium, only play a supportive role in keeping the structure open, but do not actively facilitate re-endothelialization. Materials of biological origin such as type I collagen may be more suitable as they are well known for their excellent biocompatibility [2].

Another important feature for tubular implants from biological origin would be the ability to self-expand (shape memory). Biodegradable thermally-induced shape memory tubular implants prepared from synthetic polymers have been developed and the Igaki-Tamai PLLA-based coronary stent has been successfully evaluated in humans [3]. However, alternatives prepared from materials of only natural origin are lacking.

Recently, a novel method was developed to endow collagen implants with entropy-driven shape memory using straightforward carbodiimide crosslinking in combination with compression techniques [4]. The method was used to create a star-shaped tubular scaffold with a shape memory designed for luminal closure[5]. In the current communication, we report the construction of a novel tubular implant consisting of only type I collagen, with an inverse shape-memory. This scaffold has the ability to self-expand from a crimped state to a tubular shape, similar to a self-expandable stent used in vascular disease treatment. Parameters such as deployment time, cytocompatibility and mechanical compression strength are addressed. To the best of our knowledge, this is the first self-expanding scaffold in the field of vascular medicine that consists of solely type I collagen and which may be suitable for minimally invasive implantation procedures, *i.e.* it does not require the use of open surgery with accompanying risks of infection [6].

Tubular collagen scaffolds with a luminal diameter of 4 and 6 mm were prepared from insoluble fibrillar type I collagen purified from bovine Achilles tendon by swelling the collagen (1.0%, 1.5% and 2.0% w/v) in 0.25 M acetic acid followed by a casting,

molding, freezing and lyophilization process [4, 7]. These tubular scaffolds (Figure 1, step 1) were used as the starting point for preparation of the self-expandable collagen scaffolds. Scaffolds were compressed between two aluminum objects by applying pressure under a rolling motion until the scaffold had a film-like appearance (Figure 1, step 2). Next, carbodiimide crosslinking was applied to stabilize the construct in the tubular film-like shape that it should have after implantation in a blood vessel. This step is crucial in inducing shape-memory. For crosslinking, the scaffolds were incubated in 50 mM 2-morpholineoethane sulphonic acid (MES buffer, pH 5.0, USB, Ohio, USA) containing 40% (v/v) ethanol, 33 mM N-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, Merck Schuchardt OHG, Hohenbrunn, Germany) and 6 mM N-hydroxysuccinimide (NHS, Fluka Chemie AG, Buchs, Switserland), followed by subsequent washing steps with 0.1 M Na_2HPO_4 (2 x 1h), 1.0 M NaCl (2 x 15 min), 2.0 M NaCl (2 x 15 min) and demineralized water (6 x15 min) after which they were placed in 70% (v/v) ethanol (Figure 1, step 3). Subsequently, tubular scaffolds were placed over a metal guidewire, crimped with 500 kPa using an automated radial compression machine (Blockwise Engineering, Tempe, AZ, USA) and air-dried for 15 min (Figure 1, steps 4+5).

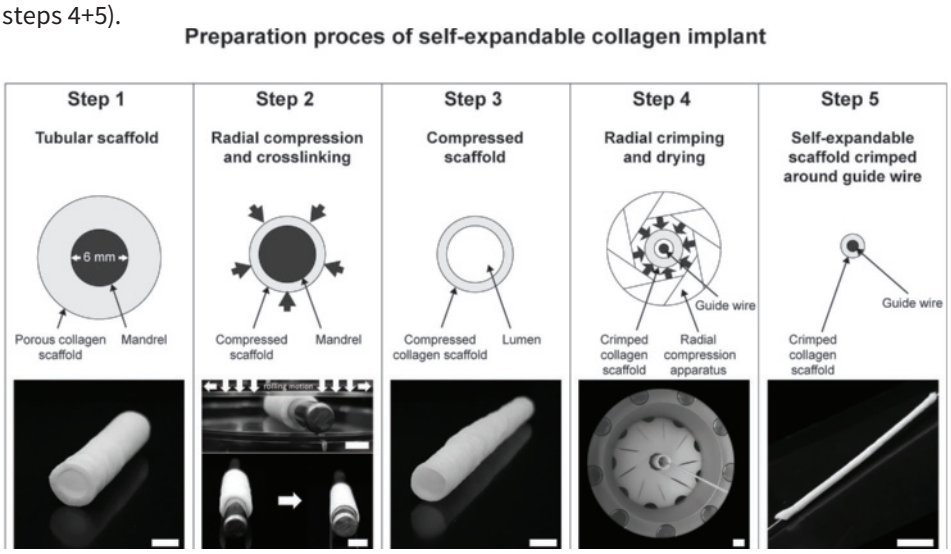


Figure 1. Production process of the self-expandable vascular implant. Step 1) Porous tubular collagen scaffolds with a luminal diameter of 6 mm (or 4 mm, not shown) were used as starting point. Step 2) The scaffolds were manually compressed between two flat objects and chemically crosslinked with the mandrel present. Step 3) Compression and crosslinking resulted in a collagen scaffold with a film-like appearance. This scaffold was used for mechanical testing. Step 4) The compressed scaffold was crimped around a metal guide wire using a automated compression machine and air-dried in crimped position. Step 5). The dry crimped collagen scaffold around a guide wire in crimped position that can expand upon exposure to water. Scale bar = 6 mm.

To visualize the self-expanding property, the crimped scaffolds with original diameters of 6 mm were placed in a plastic tube with an internal diameter of 7 mm using a metal guide wire. Water was pumped through the tube using a 50 ml syringe to allow scaffolds to expand. In this experiment collagen scaffolds made from suspensions containing 1.0%, 1.5% and 2.0% (w/v) collagen were analyzed. Videos and images were recorded using a Sony HDR-CX405 camera (Sony, Tokyo, Japan). To show that the self-expandability also works in the presence of blood plasma, the 1% scaffolds were also exposed to human blood plasma, obtained from a healthy volunteer.

To assess the ability of the tubular scaffolds to withstand compressive force, a tensile tester (Zwick/Roell Z2.5, Ulm, Germany) was used to apply force using a 20 mm square block as depicted in Figure 2C. This test simulated local compression of the scaffolds and subsequently the ability of the scaffolds to return to their original diameters was observed. A strain rate of 10 mm/min was applied until the scaffolds (6 mm diameter, 60 mm length, 1.0%, 1.5% and 2.0% (w/v) collagen) were compressed to 50% of their original diameter. A self-expandable metal (nitinol) stent with comparable dimensions was similarly compressed to make a rough comparison of compression force between our scaffolds and existing devices. Applied force and displacement were recorded.

Cytocompatiblity of three scaffolds (4 mm diameter, 1.0%, 1.5% and 2.0% (w/v) collagen) was evaluated by a cell culture experiment with human primary endothelial cells and human smooth muscle cells (Sciencecell, Carlsbad, CA, USA). In brief, scaffolds were cut to a length of 1 cm, placed in 1.5 mL Eppendorf tubes and washed with 70% (v/v) ethanol for disinfection. Next, scaffolds were washed extensively with phosphate buffered saline (PBS, pH 7.4) after which 1 mL cell suspension in endothelial growth medium-2 (Lonza, Basel, Switzerland) or smooth muscle cell medium (Sciencecell, Carlsbad, CA, USA) containing 1.3×10^5 cells was added. Seeded scaffolds were cultured overnight under rotation at 10 rpm in a 37°C incubator. After 24 h, scaffolds were transferred to 6-wells plates, cultured for 6 days, fixed with 4% paraformaldehyde in PBS and embedded in paraffin. Sections (4 µm) were stained with haematoxylin/eosin using fluoroshield mounting medium (Sigma, St Louis, MO, USA) and analyzed using a Leica DMLB microscope (H&E).

A self-expandable tubular implant was developed using a simple method based on chemical crosslinking of collagen, combined with compression and subsequent crimping of the scaffold. Upon exposure to water, the scaffold instantly deployed and

fixed to the tube wall, after which the metal guide wire could be easily removed (Movie 1, 1.0% (w/v) collagen scaffold). Deployment time increased with a higher collagen content as the 1.0% collagen scaffolds fully deployed within 15 s, followed by within 25 s and 60 for the 1.5% and 2.0% scaffolds, respectively (Figure 2A). The 1.0% scaffolds were also tested upon exposure to human blood plasma, where they fully expanded in 45 s.

When submerged in water the collagen scaffolds fully expand, and after mechanical compression in water the scaffolds always returned to their fully expanded state. Compression strength of the expanded collagen scaffolds increased with increasing collagen content; 16 mN, 29 mN and 32 mN for the 1.0, 1.5 and 2.0% (w/v) scaffolds respectively (Figure 2C). For the 1.5 and 2.0% (w/v) construct significantly more force is required for 50% compression ($p<0.001$, one-way ANOVA with Bonferroni post-hoc test). However, compared to the self-expandable nitinol stent the collagen scaffolds are relatively weak at this stage, as a force of 1 N was required for 50% compression of the nitinol stent. The cytocompatibility study showed that both cell types covered the majority of the surface on which they were seeded after six days of culturing (Figure 2B). H&E-staining showed that endothelial cells displayed an endothelial cell-like morphology. Note that due to the applied seeding procedure, both endothelial and smooth muscle cells were present on both the luminal side and the outside of the scaffold, but did not penetrate into the interior of the scaffold as shown by H&E (Figure 2B, zoomed views). The scaffold shown in Figure 2 is a 1.0% (w/v) collagen scaffold which was also representative for the 1.5 and 2.0% (w/v) as those scaffolds also showed a confluent lining. Overall these results indicate appropriate cytocompatibility.

The collagen-based, tubular-shaped implant described can be crimped or folded to a fraction of its original size, but will re-expand upon exposure to water or blood plasma. This makes it a suitable material for minimally invasive implantation techniques. Vascular medicine was chosen as an example as the use of self-expandable devices is common practice in patient care in the treatment of vascular injuries in acute (*e.g.* vessel perforation) and semi-acute (*e.g.* pseudoaneurysm) situations [8]. Vascular stents and endografts are inserted through endovascular sheaths and expanded on delivery, contact with liquids can be avoided until the construct is at the correct location. For secure positioning without displacement in a vessel with blood flow rapid expansion is essential and it is therefore advantageous that the scaffold deploys in less than one minute and presses firmly against the vessel wall to ensure maintenance of blood flow and to minimize the risk of migration of the device after implantation [9].

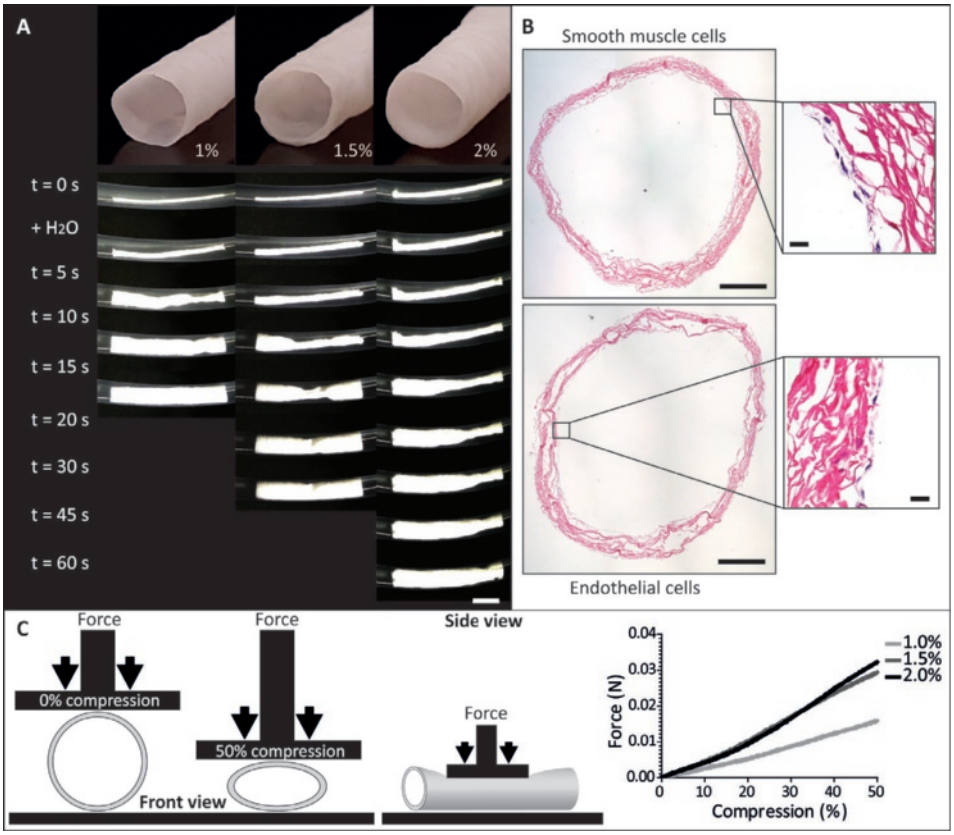


Figure 2. Scaffold deployment, cytocompatibility and mechanical characterization. A) The crimped tubular collagen scaffolds of 1.0, 1.5 and 2.0% (w/v) were exposed to water inside a plastic tube. All scaffolds deployed within 1 min, a higher collagen content resulted in a longer deployment time, scale bar is 1 cm. B) Overview of endothelial cells and smooth muscle cells seeded on 1% collagen scaffolds with enlargements. Scale bars are 1 mm and 10 µm. C) Schematic representation of the mechanical analysis (left) and the results depicted in a graph (right) showing the force needed for compression of the tubes.

A common limitation of vascular implants is the lack of re-endothelialization, thus the inability of endothelial cells to grow onto the implant. The observation that endothelial cells covered a large part of the luminal surface is promising in this respect. Cells did not migrate into the wall of the implant. This may be especially relevant for smooth muscle cells which may impair vascular healing due to excessive proliferation [10]. The observation that these cells could not enter the scaffold indicates that the collagen may provide a temporary barrier that may help to prevent intimal hyperplasia and restenosis [11]. In addition, the use of flexible collagen scaffolds may circumvent a

common limitation of metal vascular implants which is the occurrence of stress fractures due to their rigidity, restricting their use in bending points (*i.e.* groin or knee).

The shape-memory principle, which is driven by newly-established hydrophobic interactions upon crosslinking [4], may not only be relevant for treatment of vascular damage, but can also be applied for a number of other organs like bile ducts [12]. In combination with modern imaging techniques a personalized 3D-printed mold for the collagen scaffold may be created for any desired shape and application.

The main limitation of the current tubular-shaped scaffolds for both vascular and other applications is the relatively poor compression strength. Increasing collagen content in the suspension improved the compression strength. However, scaffold strength can only be increased up to a certain limit using this method. Collagen content above 2% complicates the scaffold production process because of the high viscosity of the collagen suspension. Whether this is a problem *in vivo* in the field of vascular medicine remains to be assessed: human veins also compress easily yet are successfully used as arterial conduits [13]. In case of vascular implants the stiffness of the stent must be large enough to resist migration, therefore these scaffolds require fortification and optimization for *in vivo* use. Additional methods to reinforce the collagen scaffold include the use of synthetic biodegradable polymers, or (biodegradable) metal meshes.

Collagen is often applied as a biomaterial because of its biodegradability and biocompatibility, but it has also been associated with thrombogenicity in blood vessel applications. Gelatin (denatured collagen), however, has been used to impregnate Dacron vascular grafts used in clinical practice, so as to make the material less permeable. This coating was not associated with increased thrombosis or restenosis [14]. When thrombogenicity presents as a problem, the material can be coated with heparin to prevent blood clotting using the same carbodiimide crosslinking step applied in this study [15].

In this short communication, we report the first self-expandable tubular implant consisting of solely type I collagen. It may allow implantation using minimal invasive techniques as it deploys within 60 s and is cytocompatible for vascular endothelial and smooth muscle cells. The implant may have potential for use as a biological vascular implant.

Acknowledgements

Yvonne Hoogeveen is thanked for reviewing spelling and grammar. This study was financially supported by a combined subsidiary program of the Dutch Ministry of Economic Affairs and the states of Gelderland and Overijssel (PID101020 and PID092042). The sources of funding had no other involvement in this publication.

References

[1] E. Tenekecioglu, V. Farooq, C.V. Bourantas, R.C. Silva, Y. Onuma, M. Yilmaz, P.W. Serruys, Bioresorbable scaffolds: a new paradigm in percutaneous coronary intervention, BMC cardiovascular disorders 16 (2016) 38.

[2] J. Glowacki, S. Mizuno, Collagen scaffolds for tissue engineering, Biopolymers 89(5) (2008) 338-44.

[3] S. Nishio, K. Kosuga, K. Igaki, M. Okada, E. Kyo, T. Tsuji, E. Takeuchi, Y. Inuzuka, S. Takeda, T. Hata, Y. Takeuchi, Y. Kawada, T. Harita, J. Seki, S. Akamatsu, S. Hasegawa, N. Bruining, S. Brugaletta, S. de Winter, T. Muramatsu, Y. Onuma, P.W. Serruys, S. Ikeguchi, Long-Term (>10 Years) clinical outcomes of first-in-human biodegradable poly-l-lactic acid coronary stents: Igaki-Tamai stents, Circulation 125(19) (2012) 2343-53.

[4] L.R. Versteegden, H.R. Hoogenkamp, R.M. Lomme, H. van Goor, D.M. Tiemessen, P.J. Geutjes, E. Oosterwijk, W.F. Feitz, T.G. Hafmans, N. Verdonschot, W.F. Daamen, T.H. van Kuppevelt, Design of an elasticized collagen scaffold: A method to induce elasticity in a rigid protein, Acta Biomater 44 (2016) 277-85.

[5] L.R. Versteegden, K.A. van Kampen, H.P. Janke, D.M. Tiemessen, H.R. Hoogenkamp, T.G. Hafmans, E.A. Roozen, R.M. Lomme, H. van Goor, E. Oosterwijk, W.F. Feitz, T.H. van Kuppevelt, W.F. Daamen, Tubular collagen scaffolds with radial elasticity for hollow organ regeneration, Acta biomaterialia 52 (2017) 1-8.

[6] M.J. Koens, A.G. Krasznai, A.E. Hanssen, T. Hendriks, R. Praster, W.F. Daamen, J.A. van der Vliet, T.H. van Kuppevelt, Vascular replacement using a layered elastin-collagen vascular graft in a porcine model: one week patency versus one month occlusion, Organogenesis 11(3) (2015) 105-21.

[7] J.S. Pieper, A. Oosterhof, P.J. Dijkstra, J.H. Veerkamp, T.H. van Kuppevelt, Preparation and characterization of porous crosslinked collagenous matrices containing bioavailable chondroitin sulphate, Biomaterials 20(9) (1999) 847-858.

[8] S. Jeong, Basic Knowledge about Metal Stent Development, Clinical endoscopy 49(2) (2016) 108-12.

[9] L. Xue, S. Dai, Z. Li, Biodegradable shape-memory block co-polymers for fast self-expandable stents, Biomaterials 31(32) (2010) 8132-40.

[10] T. Inoue, K. Croce, T. Morooka, M. Sakuma, K. Node, D.I. Simon, Vascular inflammation and repair: implications for re-endothelialization, restenosis, and stent thrombosis, JACC. Cardiovascular interventions 4(10) (2011) 1057-66.

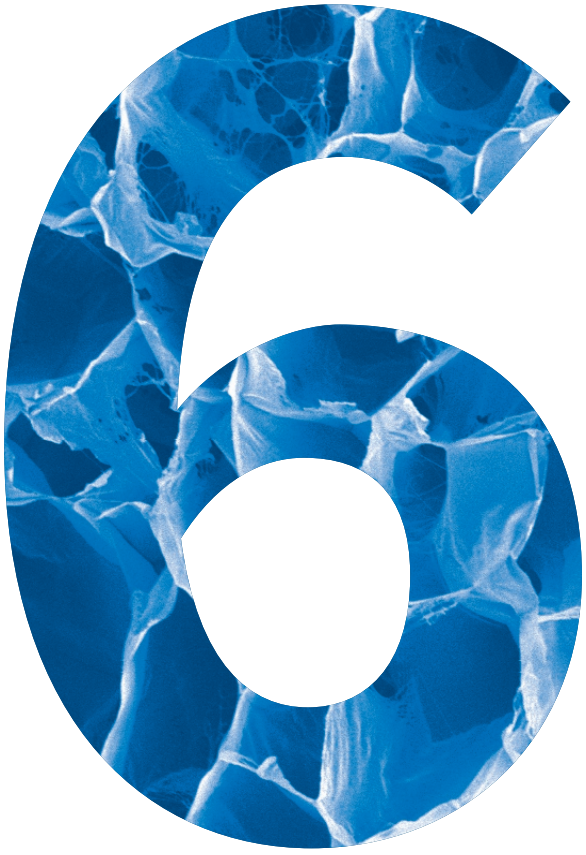
[11] T. Inoue, K. Croce, T. Morooka, M. Sakuma, K. Node, D.I. Simon, Vascular inflammation and repair: implications for re-endothelialization, restenosis, and stent thrombosis, JACC Cardiovasc. Interv. 4(10) (2011) 1057-66.

[12] D. Blero, V. Huberty, J. Deviere, Novel biliary self-expanding metal stents: indications and applications, Expert review of gastroenterology & hepatology 9(3) (2015) 359-67.

[13] C. Wilasrusmee, B. Siribumrungwong, S. Horsirimanont, N. Poprom, J. Jirasiritham, A. Thakkinstian, Clinical results of biologic prosthesis: A systematic review and meta-analysis of comparative studies, Annals of Medicine and Surgery 15 (2017) 26-33.

[14] D. Chou, A. Tulloch, D.V. Cossman, J.L. Cohen, R. Rao, G. Barmparas, J. Mirocha, W. Wagner, The Influence of Collagen Impregnation of a Knitted Dacron Patch Used in Carotid Endarterectomy, Ann. Vasc. Surg. 39 (2017) 209-215.

[15] G. Lammers, E.M. van de Westerlo, E.M. Versteeg, T.H. van Kuppevelt, W.F. Daamen, A comparison of seven methods to analyze heparin in biomaterials: quantification, location, and anticoagulant activity, Tissue engineering. Part C, Methods 17(6) (2011) 669-76.



A SALT-BASED METHOD TO ADAPT STIFFNESS AND BIODEGRADABILITY OF POROUS COLLAGEN SCAFFOLDS

Luuk R. Versteegden
Marije Sloff
Henk R. Hoogenkamp
Michiel W. Pot
Jeffrey Pang
Theo G. Hafmans
Thijs de Jong
Theo H. Smit
Sander C. Leeuwenburgh
Egbert Oosterwijk
Wout F. Feitz
Willeke F. Daamen*
Toin H. van Kuppevelt*
*contributed equally

In preparation

Abstract

Type I collagen scaffolds for tissue reconstruction often have impaired mechanical characteristics such as limited stiffness and lack of strength, resulting in a disbalance with the surrounding tissue. In this study, a new technique is presented to fine-tune stiffness and biodegradability of collagen scaffolds by treatment with concentrated salt solutions.

Collagen scaffolds were prepared by a casting, freezing and lyophilization process. Scaffolds were treated with 90% saturated salt solutions, the salts taken from the Hofmeister series, followed by chemical crosslinking. Results show that treatment with highly soluble salts consisting of a divalent cation in combination with a monovalent anion, e.g. calcium chloride, resulted in fast shrinkage of the scaffolds up to approximately 10% of the original surface area. Effective salts were mostly at the chaotropic end of the Hofmeister series. Scaffolds were evaluated microscopically, biochemically and biomechanically, and the tissue response upon *in vivo* implantation was studied. Scaffolds shrunken by $CaCl_2$ were used to evaluate salt-induced effects. Electron microscopy revealed a reduced pore size in scaffolds as well as a swollen, less organized structure of collagen fibrils. Biochemical analysis confirmed shrinkage to the level of individual collagen molecules. ICP-mass spectrometry and FTIR measurements indicated that no calcium deposits remained in the $CaCl_2$ treated scaffolds. Using nanoindentation, shrunken scaffolds were shown to be more than 10 times stiffer than non-shrunken control scaffolds (7.4×10^3 Pa vs. 4.0×10^2 Pa, with peak values up to 3.0×10^4 Pa). Subcutaneous implantation of $CaCl_2$ treated scaffolds in rats showed similar biocompatibility compared to H_2O and NaCl treated scaffolds. However, in contrast to H_2O and NaCl treated scaffolds, $CaCl_2$ treated scaffolds maintained their structural integrity without signs of major degradation after 3 months.

In conclusion, high concentrations of chaotropic salts can be used to adjust the mechanical characteristics of collagen scaffolds without affecting biocompatibility. This technique may be used in regenerative medicine to stiffen collagen scaffolds to better comply with the surrounding tissues. A molecular mechanism of the shrinkage is given.

1. Introduction

Type I collagen has been extensively used in the field of tissue engineering and regenerative medicine, particularly as a scaffolding material and as a controlled release system [1]. Its excellent biocompatibility, widespread availability and straightforward processability render collagen a very useful biomaterial [2] that has been used in many (pre)clinical studies to repair tissues like skin [3], cartilage [4] and parts of the urogenital system [5]. However, type I collagen scaffolds often show poor mechanical characteristics in applications where the scaffold is exposed to mechanical stress [6]. Hence, collagen seems more suitable for soft tissues than for hard or stiff tissue such as bone. In addition, when large defects are to be repaired, e.g. in hernia diaphragmatica, mechanical strength is often insufficient [7].

To strengthen collagen scaffolds, techniques such as chemical crosslinking using e.g. carbodiimide or glutaraldehyde are applied [8-10]. Alternatively, other components can be added to the collagen in order to enhance mechanical properties. Typically, synthetic polymers such as PLLA [11], PCL [12] or PGA [13] have been used in this respect. However, addition of other components generally complicates the preparation process, may impair biocompatibility, and imposes additional regulatory constraints.

Increasing the concentration of insoluble type I collagen in a porous scaffold is another way to enhance mechanical stiffness [14]. However, this procedure is poorly controllable for insoluble fibrillar collagen since maximum concentrations correspond to about 2% (w/v) using the currently applied preparation techniques, which is insufficient for stiff tissues.

In this study, a method is presented that enables the construction of a micro porous collagen scaffolds with a defined stiffness that display little degradation *in vivo* for at least 3 months. The method is based on fast and extensive shrinkage of collagen scaffolds by treatment with highly concentrated salt solutions. The influence of several salts selected from the Hofmeister series is assessed. The Hofmeister series of salts is a classification of anions and cations based on their ability to increase or decrease protein stability, and to influence surface tension and solubility [15]. Salt-induced shrunken scaffolds are characterized both *in vitro* and *in vivo*. The use of this method may expand the applications of collagen-based constructs in the regeneration of tissues with defined stiffness.

2. Materials and methods

2.1. Materials

N-Hydroxysuccinimide (NHS) was obtained from Fluka Chemie AG, Buchs, Switserland. Acetic acid, ammonium chloride (NH_4Cl), ammonium sulfate ($(NH_4)_2SO_4$), bovine serum albumin, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), ethanol, glutaraldehyde, hydrogen chloride, nitric acid, paraformaldehyde, sodium chloride (NaCl), sodium hydrogen phosphate ($NaHPO_4$) and sodium sulfate (Na_2SO_4) were from Merck, Darmstadt, Germany. Glycine was from Scharlau Chemicals, Barcelona, Spain. Barium perchlorate ($Ba(ClO_4)_2$), barium sulfate ($BaSO_4$), calcium chloride ($CaCl_2$), calcium sulfate ($CaSO_4$), magnesium chloride ($MgCl_2$), magnesium perchlorate ($Mg(ClO_4)_2$), 2-(*N*-morpholino)ethane sulfonic acid (MES) and trinitrobenzene sulfonic acid (TNBS) were from Sigma Aldrich, St. Louis, MO, USA. Barium chloride ($BaCl_2$) and magnesium sulfate ($MgSO_4$) were from VWR International, Radnor, PA, USA.

2.2. Methods

2.2.1. Preparation of shrunken collagen scaffolds

Preparation of porous collagen scaffolds

Type I collagen fibrils were purified from bovine Achilles tendon using extractions steps with aqueous solutions of NaCl, urea, diluted acetic acid, acetone and demineralized water [16]. Purified collagen was suspended in 0.25 M acetic acid (0.8% w/v), swollen overnight, and subsequently homogenized using a Silverson L5M-A laboratory mixer (Silverson, Chesham, UK) by mixing for 6 min at 2,500 rpm (3 min with general purpose disintegrating workhead and 3 min with a slotted workhead). Next, the suspension was deaerated using centrifugation at 100 g for 30 min. The suspension was poured into 12-well plates (1.5 mL) and 6-well plates (4 mL), frozen for 4 h at -20°C and lyophilized (Zirbus sublimator 500II, Bad Grund, Germany).

Shrinkage of scaffolds by Hofmeister series of salt treatment

To analyze the effect of different concentrated salt solutions, non-crosslinked porous scaffolds (Ø 22 mm) were exposed to 4 mL of 90% saturated salt solutions in demineralized water. Twelve salts were selected from the entire spectrum of the Hofmeister series (see Supplementary Table 1). To enhance penetration of the salt solution into the scaffold, the surface tension of 4 mL 90% saturated salt solutions was reduced by the addition of 10 µL 100% (v/v) ethanol. As a control, scaffolds were incubated in demineralized water with 10 µL ethanol. After 48 h, the surface of the

scaffolds was scanned (Epson V750 PRO) and the surface area was measured using image analysis software (ImageJ version 1.47i, NIH, Bethesda, MD, USA). For the salts that showed high shrinkage (more than 90% compared to original surface), the shrinkage was determined at different concentrations. All experiments were performed three times independently.

Shrinkage process for $CaCl_2$

We focused on $CaCl_2$, as it proved to be one of the most effective salts (see Results section). As controls, scaffolds were treated with NaCl (salt treatment, but no shrinkage) and demineralized water (H_2O). Scaffolds were shrunken in an ascending series of $CaCl_2$ to achieve gradual shrinkage. Briefly, they were first incubated for 15 min in 1 M $CaCl_2$ or 1 M NaCl, followed by 15 min 2 M, and 30 min 4 M salt solution. Control scaffolds were incubated for 30 min in demineralized water. Macroscopic images were taken and videos of the shrinking process with $CaCl_2$ were recorded with a Sony Cyber-shot DSC-H10 (Sony, Minato, Tokyo, Japan).

Stabilization of scaffolds by chemical crosslinking

After incubation in 4 M salt solution or demineralized water, scaffolds were immediately crosslinked using a carbodiimide zero-length crosslinker. In brief, scaffolds were incubated in 4 mL 50 mM MES buffer, pH 5.0, containing 40% (v/v) ethanol, 33 mM EDC and 6 mM NHS for 3 h. Next, the scaffolds were extensively washed 6 times with demineralized water to remove residual crosslinking reagents and salt. Non-crosslinked scaffolds were also washed 6 times with demineralized water. Finally, scaffolds were placed in 70% (v/v) ethanol and stored at -20°C until use.

Sterilization of the scaffolds

Scaffolds used for *in vivo* evaluation were sterilized in PBS using gamma radiation at 25 Gy from a ^{60}Co source (ISO 9001; Synergy Health BV, Ede, The Netherlands). Before sterilization, Æ 6 mm discs were cut from the scaffolds after which they were washed 6 times with PBS to remove ethanol.

2.2.2. Scaffold characterization

Scanning electron microscopy

For scanning electron microscopy, crosslinked scaffolds were washed in demineralized water to remove storage medium, and subsequently frozen at -80 °C. The frozen scaffolds were lyophilized, mounted on stubs with double-sided carbon tape, and

coated with an ultrathin gold layer (Scancoat Six Sputter Coater, Edwards, Crawley, United Kingdom). The samples were evaluated with a Sigma 300 field emission scanning electron microscope (Carl Zeiss B.V., Sliedrecht, The Netherlands) with an accelerating voltage of 10 kV. The pore sizes of the different scaffolds were determined using ImageJ analysis software.

Transmission electron microscopy

Scaffolds were fixed in 2% (w/v) glutaraldehyde in 0.1 M phosphate buffer (PB), pH 7.3 for 24 h at 4°C. After washing steps in PB, scaffolds were treated with 1% osmium tetroxide in 0.1 M PB for 1 h. Next, scaffolds were washed in PB, dehydrated in graded series of alcohol and embedded in Epon 812. Sections (1 µm) were cut, stained with toluidine blue and examined with a light-microscope (Dialux 20, Leitz). Ultrathin sections 60 nm were cut, followed by treatment with uranyl-acetate/lead-citrate for double contrast. Sections were imaged using a transmission electron microscope (JEOL 1010).

Determination of stiffness

The stiffness of $CaCl_2$, NaCl and H_2O treated collagen scaffolds were measured with the PIUMA nano-indenter (Optics11, Amsterdam, The Netherlands; Figure 3A) [17]. Before the measurement, scaffolds were incubated in PBS for 30 min followed by a passivation step of 60 min in 5% bovine serum albumin in PBS to prevent adhesion of the probe to the scaffolds.

Per sample, 25 indentions were performed in a grid of 1 x 1 mm with a distance of 200 µm between individual indentations (Figure 3B). For soft samples, an indenter probe with a stiffness of 0.05 N/m was used and for stiffer samples a probe with a stiffness of 0.47 N/m. Both probes had a tip diameter of 180 µm. The indentation depth, reached with a speed of 5 µm/s, was 15 µm and the probe was kept in place for 2 s (indentation time). Each scaffold type was measured 3 times independently.

Calcium analysis of $CaCl_2$ treated scaffolds

Two distinct methodologies were used to determine potential remnants of the $CaCl_2$ shrinking treatment. Inductively coupled plasma mass spectrometry (ICP-MS) was performed to detect elemental calcium, and Fourier transform infrared spectroscopy (FTIR) was performed to detect the presence of calcium-containing compounds which may have been formed after $CaCl_2$ treatment.

For the ICP-MS measurement, scaffolds were first hydrolyzed with 50% (v/v) nitric acid and subsequently diluted to a final concentration of 0.05 mg/ml in 1% nitric acid. Samples were analyzed using a Xseries I – ICP-MS (Thermo Fisher Scientific, Waltham, MA, USA). The total calcium content was calculated based on the measurement of the ^{43}Ca isotope as ^{40}Ca interfered with other elements. One-way ANOVA with Bonferroni post-hoc testing was performed to determine statistical differences.

Fourier Transform Infrared Spectroscopy (FTIR, Spectrum Two, Perkin Elmer, Waltham, MA, USA) was used to study the presence of calcium-containing compounds in the scaffolds. The FTIR spectra were measured in Attenuated Total Reflectance mode, with a spectral resolution of 4 cm^{-1}, a scan speed of 0.2 cm s^{-1} and 10 scans per measurement.

Differential scanning calorimetry

Differential scanning calorimetry (DSC Q1000, TA Instruments, New Castle, DE, USA) was used to assess potential denaturation of type I collagen caused by the salt treatment by measuring the denaturation temperature of non-crosslinked scaffolds treated with $CaCl_2$, NaCl and H_2O. Purified insoluble type I collagen (starting material for scaffold preparation) was used as a control. Samples (~1 mg) in aluminium pans with 25 µL PBS were heated from 1 to 80°C with a rate of 5°C/min. The endothermic peak was determined using TA Instruments Universal Analysis 2000 software (Version 4.5A).

Gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to assess potential denaturation of collagen in the scaffold after salt treatment. Briefly, 2-3 mg of non-crosslinked collagen scaffolds was digested overnight at ambient temperature in 75 µL 0.25 M acetic acid containing 3 mg/mL pepsin after which an equal volume of a 2-fold concentrated non-reducing sample buffer was added. Samples and controls were loaded on an 8% (w/v) polyacrylamide gel without prior heating. After running at 150 V, the gel was stained with 0.1% (w/v) Coomassie Brilliant Blue solution.

Evaluation of the degree of crosslinking

The degree of crosslinking of the salt treated scaffolds was calculated by assaying the loss of primary amine groups after crosslinking using a TNBS assay [18]. After salt treatment, crosslinking and rinsing steps with water, the scaffolds were lyophilized and subsequently incubated for 30 min in 4% (w/v) $NaHPO_4$ at 22 °C. Subsequently, samples were incubated for 2 h at 40 °C in 0.5% (w/v) TNBS in demineralized water, after

which the samples were hydrolyzed with 6 M HCl for 1.5 h at 60 °C. Absorbance at 420 nm was measured with a spectrophotometer (Bio-Tek, Bad Friedrichshall, Germany). Glycine was used for the calibration curve. Non-crosslinked controls for each condition were used to calculate the percentage of crosslinked amine groups. Experiments were performed in triplicate in three different experiments.

2.2.3. In vivo evaluation of salt treated scaffolds in a rat model

The study was approved by the Ethical Committee on Animal Research of the Radboud University Nijmegen, The Netherlands (RU-DEC 2013-144) and executed according to Dutch legislation and guidelines for the use of laboratory animals.

Nine male Wistar rats (Harlan Laboratories, Horst, The Netherlands) of 3 months old (~400 g) were housed in trios in a controlled environment (19-21°C, 12-12 h day/night cycle) with *ad libitum* access to food and water.

Using Microsoft Excel, the rats were randomly divided over three experimental groups. Rats were anesthetized with 2-3% (v/v) isoflurane, and received buprenorphine by intramuscular injection as analgesic. Three different scaffolds (a $CaCl_2$ treated scaffold, a NaCl treated scaffold, and a H_2O treated scaffold) were randomly implanted in three different subcutaneous pockets created on the back of each rat. At the time of implantation, researchers performing evaluations were blinded for the type of scaffold by naming them A, B and C. Reference sutures (Prolene 5.0, Ethicon, Somerville, NJ) were placed next to the scaffold. Wounds were sutured using Prolene 5.0 and for reinforcement of the sutures hog rings were placed over the wound. Immediately after surgery and 12, 24, 36 and 48 h later, rats received additional buprenorphine via intramuscular injection. During the whole experiments the general health condition of the rats was monitored. At 3 days (group 1), 14 days (group 2) and 90 days (group 3) after implantation the animals were sacrificed using carbon dioxide gas. Scaffolds were excised from the subcutaneous pockets, fixated using 4% (w/v) paraformaldehyde in phosphate buffer (pH 7.4) and embedded in paraffin. Sections (5 µm) were obtained using a HM 340E Electronic Rotary Microtome (Thermo Fisher Scientific, Waltham, MA, USA). Tissue sections were stained with haematoxylin/eosin and Elastin Von Masson. To visualize calcifications, a Von Kossa and an Alizarin red staining were performed. Blinding was applied for the animal experiments; however, at time of evaluation the blinded feature of this study was lost due to the distinct characteristics of each scaffold type.

3. Results

3.1. Effect of salts from the Hofmeister series on collagen scaffolds

To evaluate the effect of the Hofmeister salts on porous disc-shaped collagen scaffolds, scaffolds were incubated for 48 h in 90% saturated solutions. For a number of salts (e.g. $CaCl_2$) large shrinkage of the scaffolds was observed, whereas other salts caused intermediate, minor or no shrinkage (Figure 1). The surface area was shrunken for more than 90% in case of $CaCl_2$ (93±1%, Figure 1A), $Ba(ClO_4)_2$ (91±1%), $MgCl_2$ (93±1%), and $Mg(ClO_4)_2$ (94±1%) (Figure 1B). The process was extremely fast: within 30 s the scaffold had almost completely shrunken (Movie 1). Salts that caused only limited shrinkage were $MgSO_4$ (8±11%), Na_2SO_4 (13±7%), NaCl (15±8%), NH_4Cl (20±9%), $CaSO_4$ (28±14%), and $BaCl_2$ (39±8%). $(NH_4)_2SO_4$ and $BaSO_4$ displayed no shrinkage. In general, salts containing divalent cations in combination with monovalent anions, resulted in major shrinkage of the scaffold.

Focussing on salts that induced shrinkage of >90%, we studied the minimal concentration required to achieve full shrinkage of the scaffold (Figure 1C). For $Mg(ClO_4)_2$ and $Ba(ClO_4)_2$ this was 1 M, whereas for $CaCl_2$ and $MgCl_2$ it was 1.5 and 3 M, respectively. Being one of the most effective salts for shrinkage, $CaCl_2$ was selected for further studies, and we compared this salt treatment to treatment using NaCl and H_2O only.

3.2. Morphological characterization

Scaffolds treated with $CaCl_2$, NaCl and H_2O were characterized with scanning and transmission electron microscopy. Cross-sectional images revealed that salt treated scaffolds remained porous (Figure 2, left panels). $CaCl_2$ treated scaffolds displayed circular pores but, compared to the H_2O treated scaffold, the pore size decreased from 40 – 120 µm to 20 – 60 µm. In the NaCl treated scaffolds, the pore structure had somewhat flattened and the size of pores was slightly decreased.

$CaCl_2$ treatment had a distinct swelling effect on the individual collagen fibrils in the scaffolds. The fibrils in the pore wall of $CaCl_2$ treated scaffolds appeared distended, resulting in the loss of the characteristic banding pattern, whereas clear striation patterns were visible in NaCl and H_2O treated scaffolds (Figure 2B-C, right panels).

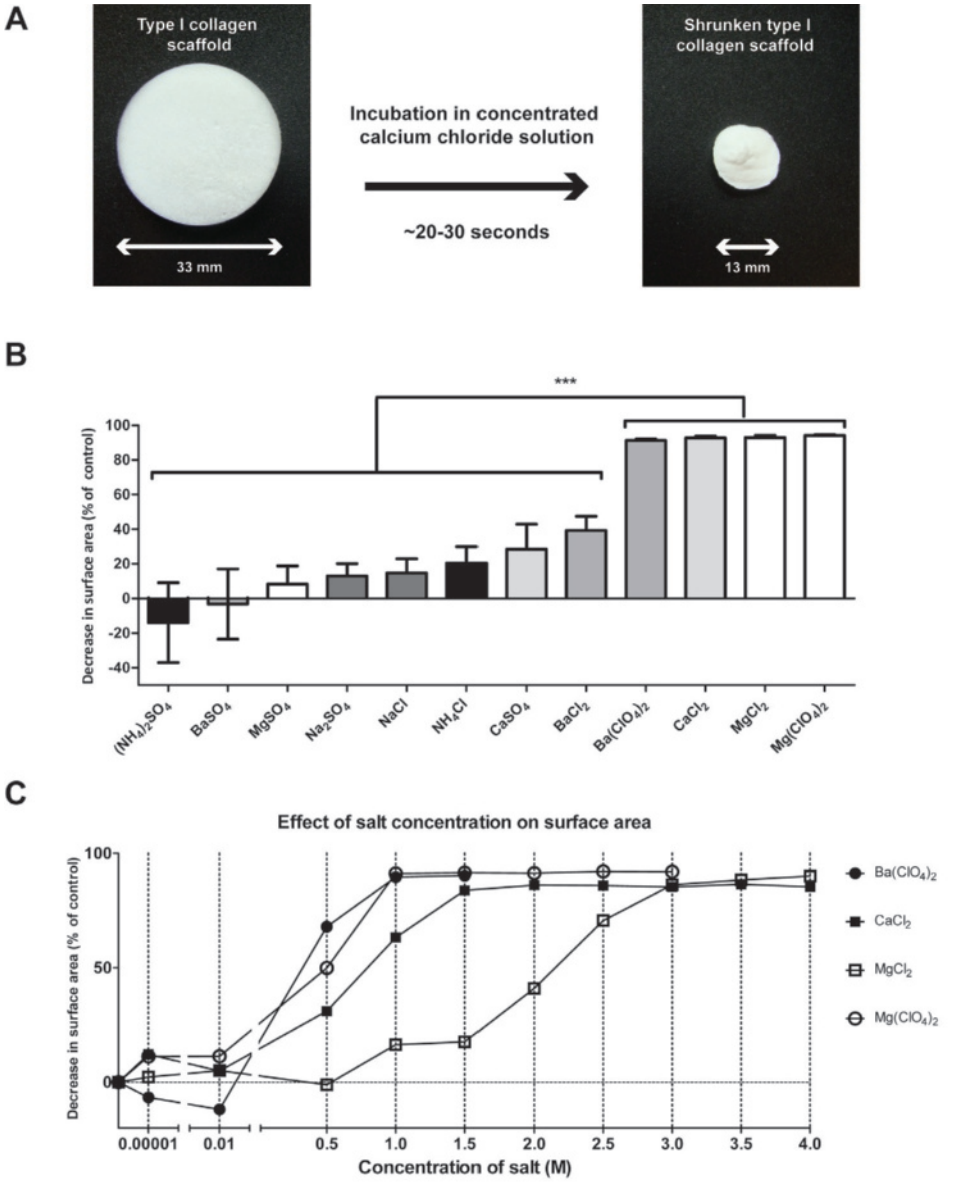


Figure 1: The shrinkage effect of different salts from the Hofmeister series on 0.8 % (w/v) collagen scaffolds. A) Type I collagen scaffold before and after exposure to 4 M $CaCl_2$. B) The effect of 90% saturated salt solutions (x-axis) on the surface area of on collagen scaffolds. Note that $Ba(ClO_4)_2$, $CaCl_2$, $MgCl_2$, and $Mg(ClO_4)_2$ induced shrinkage to over 90% compared to control scaffolds, n=3. Statistical analysis: One-way ANOVA with Bonferroni post-hoc test, $p<0.001$. Note: due to variability in solubility, the 90% saturated salt solutions vary in molarity (see Supplementary Table 1). C) The effect of salt concentration (x-axis) on the degree of shrinkage (y-axis) for a subset of salts that showed over 90% shrinkage in Figure 1B, n=3.

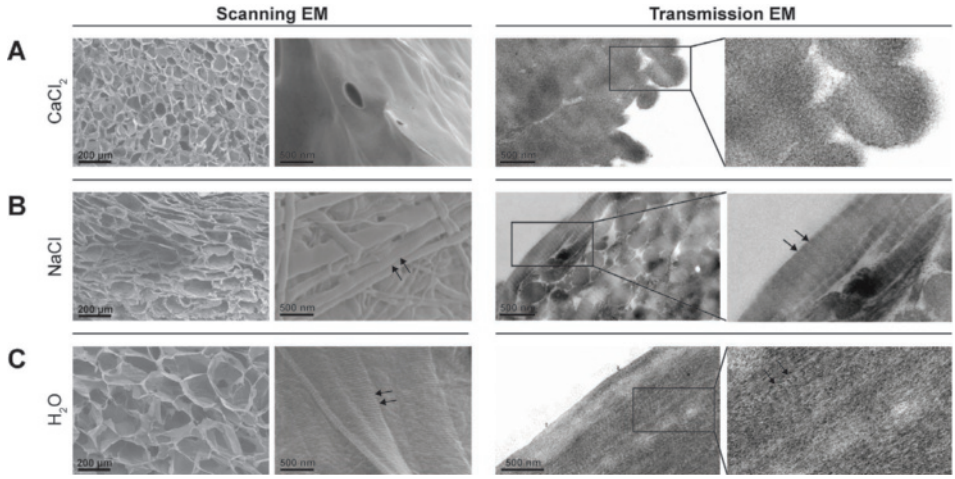


Figure 2: Morphological characterization of collagen scaffolds. Scanning and transmission electron microscopical images of A) a $CaCl_2$ treated scaffold showing the altered pore structure and the swollen fibrils without banding pattern; B, C) NaCl B) and H_2O C) treated scaffolds showing the characteristic striation of collagen fibrils indicated with black arrows.

3.3. Mechanical characterization

The shrinking process may have an effect on the mechanical properties of the scaffolds due to the increased collagen content per volume. Therefore, the stiffness was assessed using nanoindentation (Figure 3A). $CaCl_2$, NaCl and H_2O treated scaffolds were measured three times independently with 25 indentations on different locations per scaffold (Figure 3B). $CaCl_2$ scaffolds (7.4×10^3 Pa) were stiffer compared to the NaCl (1.2×10^3 Pa) and the H_2O treated scaffolds (4.4×10^2 Pa). Peak values even showed a stiffness of more than 3.0×10^4 Pa, most likely when the tip indented exactly on top of a pore wall indicating that the stiffness of $CaCl_2$ treated collagen material is even higher than 7.4×10^3 Pa.

3.4. Presence of calcium or calcium-containing compounds in collagen scaffolds

Inductively coupled plasma mass spectrometry showed that the $CaCl_2$ treated scaffold contained only 17.8±5.2 ppb calcium corresponding to 0.36±0.03 ng per µg scaffold (Table 1). The NaCl and H_2O treated scaffolds contained 0.25±0.02 and 0.29±0.1 ng calcium per µg scaffold respectively, which were both not significantly different from the $CaCl_2$ treated scaffold (one-way ANOVA with Bonferroni post-hoc testing, $p>0.05$). This suggests that no remnants of the calcium treatment were present in the $CaCl_2$

shrunken scaffolds. Using Fourier-transform infrared spectroscopy no absorption peaks were detected that could be attributed to calcium-containing compounds (Table 1).

3.5. Degree of crosslinking

The degree of crosslinking in the $CaCl_2$, NaCl, and H_2O treated scaffolds was estimated by loss of primary amine groups (Figure 4). Non-crosslinked $CaCl_2$, NaCl, and H_2O treated scaffolds contained 241±41, 244±43, 246±37 nmol of primary amine groups per mg collagen, respectively, whereas crosslinked scaffolds had 81±12, 111±8 and 139±10 nmol. The reduction of primary amine groups was 66% for the $CaCl_2$ treated scaffold, 55% for the NaCl treated scaffold and 42% for the H_2O treated scaffold (Figure 4A). The difference in crosslinking percentage between the three scaffolds types were statistically different as assessed by a one-way ANOVA with Bonferroni post-hoc testing, $p<0.05$.

3.6. Degree of denaturation

Electron microscopical images of $CaCl_2$ treated scaffolds displayed swollen fibrils which may be caused by partial denaturation induced by $CaCl_2$. Differential scanning calorimetry showed that denaturation temperature was reduced from 58±2°C (type I collagen) to 47±1°C after calcium treatment indicating denaturation (Figure 4B). The denaturation temperature of the H_2O (59±1°C) and NaCl (59±1°C) treated scaffolds did not change significantly from purified insoluble type I collagen.

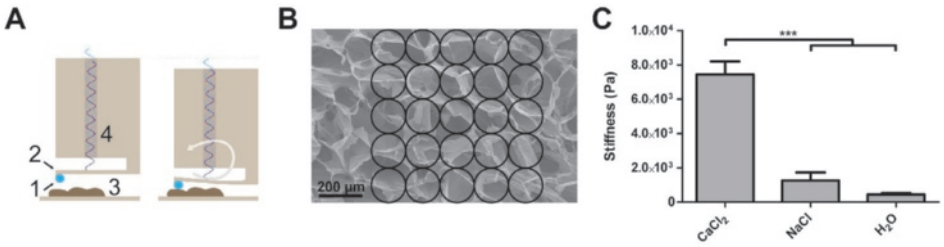


Figure 3. Mechanical characterization using nanoindentation. A) Working mechanism of PIUMA nanoindentation equipment: a probe (1) connected to a cantilever (2) indents the sample (3) and bends through. The degree of bending is measured via an optical cable (4) and converted to the degree of stiffness. B) Locations of indentation on the scaffold visualized with a representative SEM image. C) The stiffness of $CaCl_2$, NaCl and H_2O treated scaffolds measured with a PIUMA nanoindentation apparatus. Each condition was tested in triplicate with 25 indentations per scaffold. Bars represent mean ± standard error of the mean. One way ANOVA with Bonferroni post-hoc test, *** $p<0.0001$.

SDS-PAGE substantiated that $CaCl_2$ treatment induced denaturation (Figure 4C). When insoluble type I collagen is not denatured, no bands will be visible on an SDS-PAGE gel, as shown by the control samples (H_2O and NaCl treated scaffolds). The $CaCl_2$ treated collagen showed clear collagen α-chain bands (black arrows) indicating denaturation of the insoluble type I collagen fibrils. In the lanes loaded with pepsin treated samples, α, β, and γ bands were detected for H_2O and NaCl treated scaffolds (see red arrows in Figure 4C), while for $CaCl_2$ treated scaffolds a smear was observed. Pepsin usually does not cleave intact type I collagen triple helices, but if the helix is partially denaturated it will become susceptible for pepsin cleavage over the entire helix, resulting in a smear. Consequently, the results of the SDS-PAGE indicate that $CaCl_2$ induced denaturation of the triple helix.

3.7. In vivo biocompatibility

All rats survived surgery without complications and did not seem to experience any discomfort caused by the subcutaneously implanted scaffolds throughout the entire study. The surgical wounds healed normally.

In the rats sacrificed 3 days after implantation, the original shape of all scaffolds was maintained, but they were covered with a macroscopically visible layer of fibrous tissue. Visual signs of inflammation were absent, but histologically a mild immune

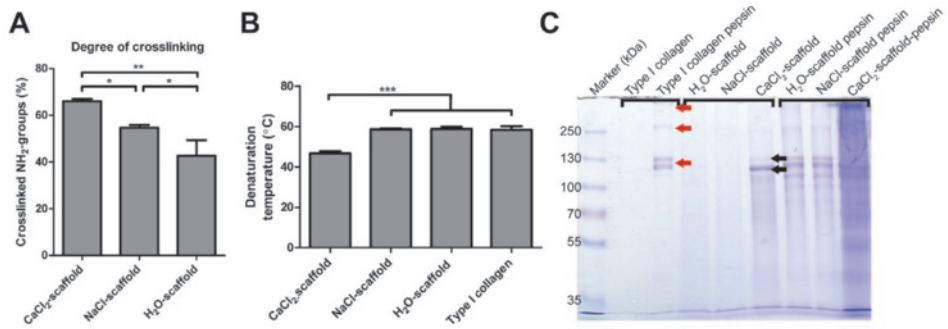


Figure 4. Biochemical and biophysical characterization of $CaCl_2$, NaCl and H_2O treated scaffolds. A) Degree of crosslinking (TNBS-assay) indicating differences between scaffolds (n=3, one–way ANOVA with Bonferroni post-hoc test, *p<0.05, **p<0.01). B) Denaturation temperature (T_d) of purified insoluble type I collagen and various scaffold types indicated that $CaCl_2$ treatment lowered the T_d (n=6, one–way ANOVA with Bonferroni post-hoc test, ***P<0.001). C) SDS-PAGE gel of purified insoluble type I collagen, $CaCl_2$, NaCl and H_2O treated scaffolds with and without pepsin treatment indicating that $CaCl_2$ treatment causes denaturation of the triple helix. The red arrows indicate the location of α, β, and γ bands after pepsin digestion and the black arrows indicates the location of α bands after $CaCl_2$ treatment.

response was observed for all scaffolds based on the presence of granulocytes and macrophages. For the $CaCl_2$ treated scaffolds, granulocytes and giant cells were located at the rims of the scaffold (Figure A.1).

In the rats sacrificed 14 days after implantation, all scaffolds were partially encapsulated and upon excision it appeared that the H_2O and NaCl treated scaffolds had lost their original shape, while the $CaCl_2$ treated scaffolds looked intact. Microscopic analysis showed increased cellular infiltration in H_2O and NaCl treated scaffolds, which may contribute to the deformation, possibly by biodegradation of the scaffold (Figure A.1).

In the rats sacrificed 90 days after implantation scaffolds treated with H_2O and NaCl showed to be deformed indicating degradation, whereas the $CaCl_2$ treated scaffolds still looked intact. All scaffolds were fully encapsulated as can be observed in the H&E staining in Figure 5. H&E and Elastin von Masson (EvM) stainings indicated that cells (giant cells, monocytes, macrophages and fibroblasts) infiltrated the H_2O and NaCl treated scaffolds (Figure 5B+C, left and middle panel). Cell migration was limited for the $CaCl_2$ treated scaffolds as cells were only observed in the outer edges of the scaffold (Figure 5A, left and middle panel). For all scaffolds the immunological reaction was mild. Alizarin Red and Von Kossa stainings indicated the absence of calcium/calcification in the implanted scaffolds (Figure 5C, right panels). See Figure A.2 for positive controls (human bone tissue).

4. Discussion

4.1. Scaffold properties

Over the years, methods to enhance stiffness of insoluble type I collagen scaffolds have been widely investigated. Generally, crosslinking techniques are applied to stabilize collagen scaffolds, which increase their overall strength, cohesion and stiffness [9]. However, for a number of applications this is not sufficient and (synthetic) polymers have been added to further improve mechanical properties [19]. In this study, a simple method is described to adapt the stiffness of collagen scaffolds and increase their strength. The method is based on treatment with a concentrated salt solution resulting in major shrinkage of the scaffolds, thus increasing the collagen concentration in the walls of the porous constructs. The method resulted in rigid collagen scaffolds that were more than 10 times stiffer than untreated scaffolds, while *in vivo* biocompatibility was maintained.

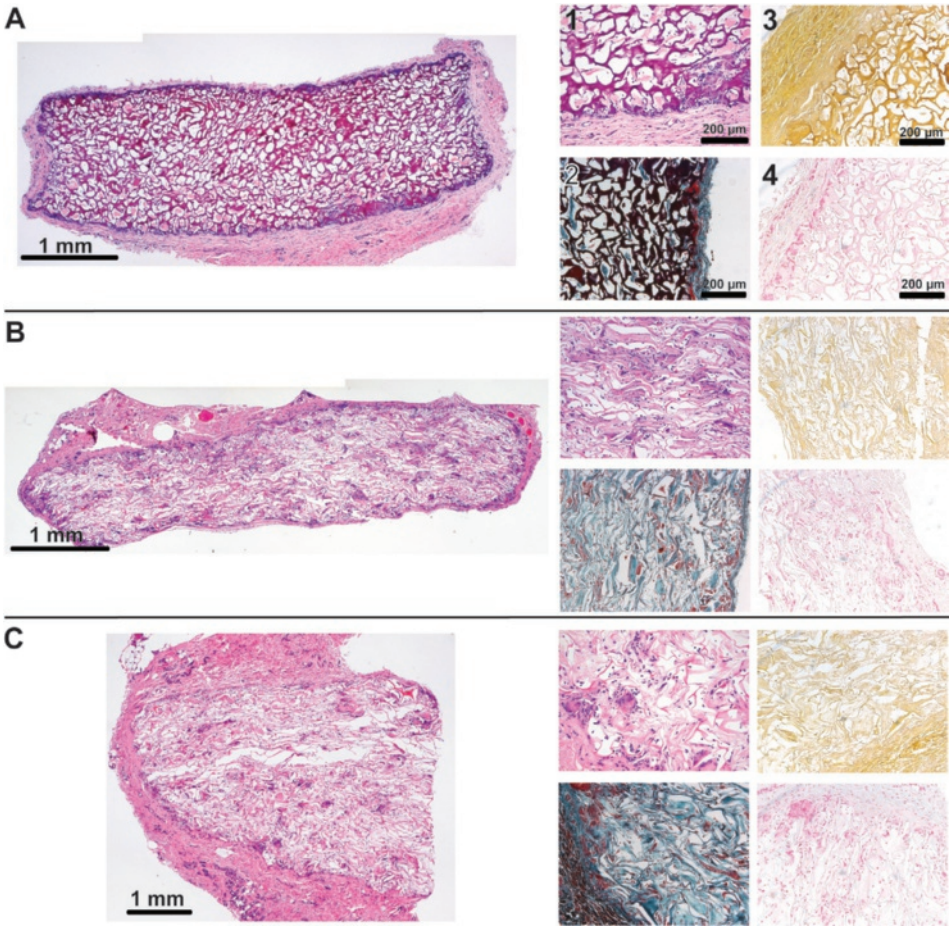


Figure 5: Biocompatibility of $CaCl_2$, NaCl, and H_2O treated scaffolds study in a subcutaneous rat model. In the left panel H&E overview images are depicted of a $CaCl_2$ treated scaffold A), a NaCl treated scaffold B), and a H_2O treated scaffold C) 90 days after implantation. On the right, enlarged images of similar magnification are shown for every scaffold type. Image 1: H&E staining, 2: Elastin von Masson staining, 3:Alizarin Red staining, 4:Von Kossa staining.

By adjusting the type and concentration of the salt used, the method may allow the construction of scaffolds with a specific stiffness. This is relevant since every tissue in the human body has its own characteristic stiffness caused by a distinct composition of the extracellular matrix. In recent years it has become clear that stiffness of the extracellular matrix plays an important role in differentiation of stem cells [20]. Consequently, to achieve optimal tissue regeneration it is important to take the stiffness of the target tissue into account when designing biomaterials. Current applications of collagen scaffolds are often limited to soft tissues, e.g. skin. Scaffolds

treated with the shrinking method showed to have an average stiffness of 7.4 x 10^3 Pa with peak values of 3.0 x 10^4 Pa. Consequently, this may expand the application range of collagen scaffolds to harder tissues such as muscle (~7-18 kPa), cartilage (~25 kPa) and unmineralized bone (~35 kPa) [21, 22].

4.2. Molecular mechanism of shrinkage

To investigate the molecular mechanism responsible for the shrinkage, the effect of salts from the Hofmeister series on collagen scaffold was analyzed. Only highly concentrated salts with divalent cations (Ca^{2+}, Mg^{2+} and Ba^{2+}, all at the chaotropic site of the series)

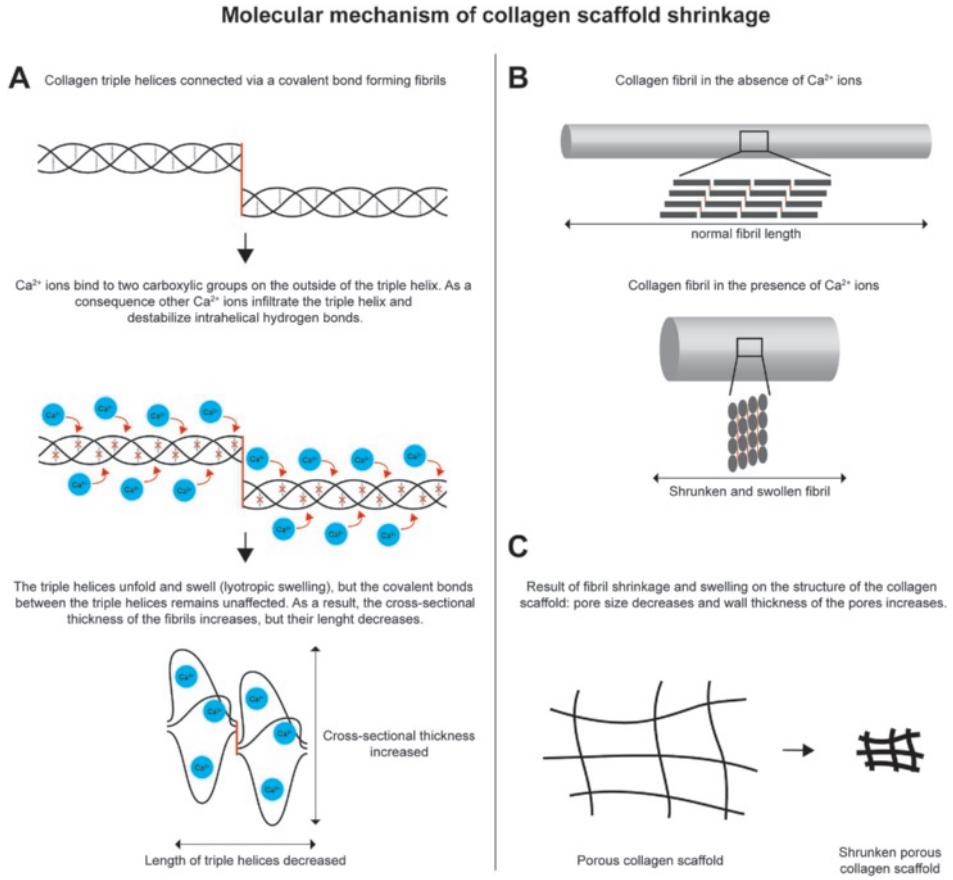


Figure 6. Schematic representation of proposed molecular mechanism of shrinkage. A) Ca^{2+} ions destabilize the triple helix by first binding to carboxylic groups and secondly by disrupting the hydrogen bonds inside the triple helix resulting in swelling of the triple helix, making it thicker but shorter. B) The fibril structure changes dramatically after addition of Ca^{2+} ions: they become thicker and shorter. C) The thicker and shorter fibrils result in shrinkage of the collagen scaffold.

in combination with an anion (not being sulfate) were able to induce shrinkage up to 90% of the initial surface area. EM images of shrunken collagen scaffolds showed that fibrils were swollen and had lost their characteristic banding pattern. A phenomenon described in leather research as 'lyotropic swelling', may explain the shrinkage [23]. During lyotropic swelling, cations such as Ca^{2+} disrupt the hydrogen bonds that stabilize the triple helical structure. The cations interact with negatively charged carboxylic groups, thereby attracting water inside the helix, causing it to swell. For collagen, we propose the mechanism displayed in Figure 6. Type I collagen molecules consist of 2 α_1 and 1 α_2 polypeptide chains, which are very close together (triple helix conformation), and are shifted one amino acid with respect to each other. This brings the carboxylic groups of the Glu and Asp amino acid residues in e.g. two α_1 chains (~100 Glu/Asp per chain) in close proximity to each other. Bivalent cations such as Ca^{2+} bind very well to two close carboxylic groups, and this may be an initiating event in the denaturation of the triple helix and the subsequent swelling process. In contrast to reversible collagen swelling with 0.25 M acetic acid at pH 2.5 where fibrils start to repel each other because they have a similar charge due to the pH effect, lyotropic swelling takes place inside the triple helix and is irreversible [24]. When triple helices are exposed to high concentrations of cations such as Ca^{2+}, the triple helix gets disrupted (Figure 6A, upper panel) causing the helix to swell. As a consequence, additional Ca^{2+} can enter the inner core of the helix and disrupt the hydrogen bonds that stabilize the helix structure resulting in (partial) denaturation of the triple helix. However, the (natural) covalent bonds between the individual triple helices are not affected (Figure 6A, middle panel), preventing the fibril to fall apart. Consequently, as the helices swell, they become thicker and shorter (Figure 6A, lower panel). On the level of fibrils this will lead to swollen but shortened fibrils (Figure 6B). When this occurs throughout the scaffold it will result in an increased thickness of the pore walls and reduced pores sizes ultimately leading to shrinkage and stiffening of the entire scaffold. This process is irreversible and it was observed that the scaffolds remain shrunken upon removal of $CaCl_2$.

4.3. Biocompatibility and potential applications

Type I collagen has shown to be a biocompatible scaffolding material in many studies [25] also in combination with EDC/NHS crosslinking [26, 27]. After $CaCl_2$ treatment the collagen triple helix was partly denaturated as indicated by a decrease in denaturation temperature and the appearance of (pepsin-sensitive) α-chains on SDS-PAGE. However, subcutaneous implantations of the $CaCl_2$ treated scaffold revealed a similar cellular response as observed for H_2O and NaCl treated scaffolds indicating proper

biocompatiblity. The major difference was that the $CaCl_2$ treated scaffolds were barely infiltrated by cells explaining that the scaffolds were still looked intact 90 days after implantation maintaining their morphology. Biodegradability was clearly decreased and this may open ways to prepare collagen-based scaffold with long-term integrity.

The method developed to stiffen collagen scaffolds opens new opportunities in the field of regenerative medicine. By manipulating a number of parameters such as collagen concentration, $CaCl_2$ concentration or shrinkage time, scaffolds with varying stiffness may be created. The preparation of such a wide stiffness range may serve as an *in vitro* platform for stem cell research to further investigate the role of stiffness on stem cell differentiation in a natural 3D extracellular matrix. Currently, this role is generally evaluated using synthetic hydrogels prepared from e.g. polyacrylamide [20, 28, 29]. A 3D environment created from a natural component of the human extracellular matrix would resemble the native stem cell niche more closely and may be advantageous to further elucidate the molecular mechanism involved in tissue regeneration and stem cell differentiation.

The *in vivo* results indicated that 90 days after implantation the $CaCl_2$ treated scaffolds were intact, not deformed by surrounding tissue, and hardly populated by cells in contrast to untreated scaffolds. The $CaCl_2$ treated scaffolds seemed to act as a very slow degrading implant maintaining their morphological and mechanical properties over a long time period. In this respect, a function as a reservoir facilitating sustained release of drugs (e.g. anti conception) or antigens (immunization) may be anticipated. The biocompatibility in combination with the slow degradation properties of $CaCl_2$ treated scaffolds may be interesting as a coating for medical implants such as glucose sensors as shown in a study by Yu *et al.* [30].

5. Conclusions

In this study, a method was developed to enhance stiffness of porous scaffolds prepared from type I collagen using concentrated salt solutions, as exemplified by $CaCl_2$. The observed shrinking effect, likely driven by the disruption of stabilizing hydrogen bonds in the triple helix, resulted in stiffer scaffolds with retention of microporosity, with reduced biodegradability, and similar biocompatibility compared to control scaffolds. The method may be useful for the construction of collagen scaffolds with stiffness in line with the tissue to be regenerated, but also e.g. as a slow drug delivery system.

6. Acknowledgements

The technicians from the Central Animal Laboratory, Radboud university medical center are acknowledged for their assistance with the animal experiments. Patricia Hoekstra (Marel Townsend Further Processing) is acknowledged for support with DSC measurements and analysis. This project was financed by PIDON (NovioTissue project), a subsidiary program of the Dutch Ministry of Economic Affairs, and the states of Gelderland and Overijssel (PID101020).

Table 1. Presence of calcium in the $CaCl_2$, NaCl and H_2O treated scaffolds

Scaffold type	ICP-MS Calcium content (ng calcium per µg collagen scaffold)	FT-IR Presence of calcium-containing compounds
$CaCl_2$ scaffold	0.36 ± 0.03	None
NaCl scaffold	0.25 ± 0.02	None
H_2O scaffold	0.29 ± 0.1	None

7. Appendix

Supplementary Table 1: Hofmeister series of salts used in this study.

Salts	Maximum solubility in water (g/L)	90% saturated solution (g/L)	Molarity of 90% saturated solution (mol/L)	Decrease in surface area (% of control)
NH_4Cl	395 [1]	355.5	7.38	20
$(NH_4)_2SO_4$	764 [1]	687.6	5.78	-14
$BaCl_2$	370 [1]	333	1.78	39
$Ba(ClO_4)_2$	1985 [1]	1786.5	5.90	91
$BaSO_4$	0.0031 [1]	0.00279	1.33E-05	-3
$CaCl_2$	745 [1]	670,5	6.71	93
$CaSO_4$	3.0 [1]	2.7	0.022	28
$MgCl_2$	543 [1]	488.7	5.70	93
$Mg(ClO_4)_2$	993 [1]	893.7	4.45	94
$MgSO_4$	360 [1]	324	2.99	8
NaCl	360 [1]	324	6.16	15
Na_2SO_4	195 [2]	175.5	1.37	13

Data on maximum solubility in water were derived from [1]PubChem (National Center for Biotechnology, National Institute of Health, USA), and [2]The Solubility Table of Wikipedia, the Free Encyclopedia.

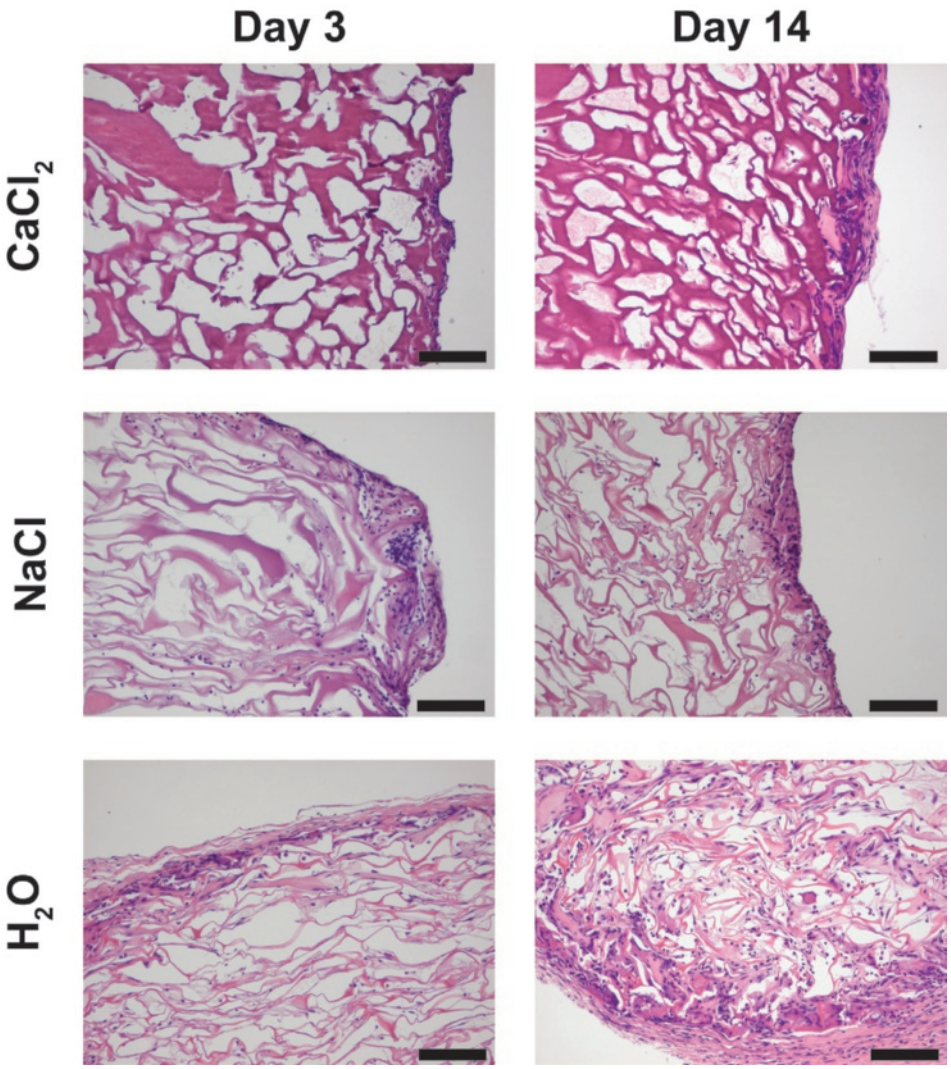


Figure A.1. Biocompatibility of $CaCl_2$, NaCl, and H_2O treated scaffolds study in a subcutaneous rat model. H&E images of a $CaCl_2$ treated scaffold, a NaCl treated scaffold, and a H_2O treated scaffold 3 days after implantation (left panel) and 14 days after implantation (right panel). Scale bars are 200 µm.

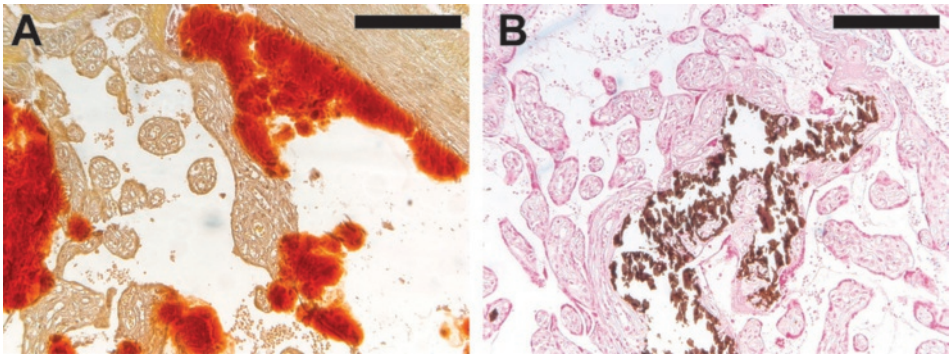


Figure A.2. Positive control used to evaluate experimental samples using the Von Kossa and Alizarin Red staining. A) Bone tissue staining with Alizarin Red in which calcium is visible in red. B) Von Kossa staining of bone tissue in which calcium phosphate is visible in brown. Scale bars are 200 µm.

8. References

[1] S. Gomes, I.B. Leonor, J.F. Mano, R.L. Reis, D.L. Kaplan, Natural and Genetically Engineered Proteins for Tissue Engineering, Prog Polym Sci 37 (2012) 1-17.

[2] C.H. Lee, A. Singla, Y. Lee, Biomedical applications of collagen, Int J Pharm 221 (2001) 1-22.

[3] A.A. Chaudhari, K. Vig, D.R. Baganizi, R. Sahu, S. Dixit, V. Dennis, S.R. Singh, S.R. Pillai, Future Prospects for Scaffolding Methods and Biomaterials in Skin Tissue Engineering: A Review, Int J Mol Sci 17 (2016).

[4] E.A. Makris, A.H. Gomoll, K.N. Malizos, J.C. Hu, K.A. Athanasiou, Repair and tissue engineering techniques for articular cartilage, Nat Rev Rheumatol 11 (2015) 21-34.

[5] L.R. Versteegden, P.K. de Jonge, J. IntHout, T.H. van Kuppevelt, E. Oosterwijk, W.F. Feitz, R.B. de Vries, W.F. Daamen, Tissue Engineering of the Urethra: A Systematic Review and Meta-analysis of Preclinical and Clinical Studies, Eur Urol (2017).

[6] A. George, S. Ravindran, PROTEIN TEMPLATES IN HARD TISSUE ENGINEERING, Nano Today 5 (2010) 254-266.

[7] K.M. Brouwer, W.F. Daamen, H.R. Hoogenkamp, P.J. Geutjes, I. de Blaauw, W. Janssen-Kessels, W. de Boode, E. Versteeg, R.M. Wijnen, W.F. Feitz, M. Wijnen, T.H. van Kuppevelt, Collagen-Vicryl scaffolds for reconstruction of the diaphragm in a large animal model, J Biomed Mater Res B Appl Biomater 102 (2014) 756-63.

[8] G.P. Huang, S. Shanmugasundaram, P. Masih, D. Pandya, S. Amara, G. Collins, T.L. Arinzeh, An investigation of common crosslinking agents on the stability of electrospun collagen scaffolds, J Biomed Mater Res A 103 (2015) 762-71.

[9] L.H. Olde Damink, P.J. Dijkstra, M.J. van Luyn, P.B. van Wachem, P. Nieuwenhuis, J. Feijen, Cross-linking of dermal sheep collagen using a water-soluble carbodiimide, Biomaterials 17 (1996) 765-73.

[10] M.W. Pot, K.A. Faraj, A. Adawy, W.J. van Enckevort, H.T. van Moerkerk, E. Vlieg, W.F. Daamen, T.H. van Kuppevelt, Versatile wedge-based system for the construction of unidirectional collagen scaffolds by directional freezing: practical and theoretical considerations, ACS Appl Mater Interfaces 7 (2015) 8495-505.

[11] H. Lu, H.H. Oh, N. Kawazoe, K. Yamagishi, G. Chen, PLLA-collagen and PLLA-gelatin hybrid scaffolds with funnel-like porous structure for skin tissue engineering, Sci Technol Adv Mater 13 (2012) 064210.

[12] S.J. Lee, J. Liu, S.H. Oh, S. Soker, A. Atala, J.J. Yoo, Development of a composite vascular scaffolding system that withstands physiological vascular conditions, Biomaterials 29 (2008) 2891-8.

[13] S. Toosi, H. Naderi-Meshkin, F. Kalalinia, M.T. Peivandi, H. HosseinKhani, A.R. Bahrami, A. Heirani-Tabasi, M. Mirahmadi, J. Behravan, PGA-incorporated collagen: Toward a biodegradable composite scaffold for bone-tissue engineering, J Biomed Mater Res A 104 (2016) 2020-8.

[14] C.M. Tierney, M.G. Haugh, J. Liedl, F. Mulcahy, B. Hayes, F.J. O'Brien, The effects of collagen concentration and crosslink density on the biological, structural and mechanical properties of collagen-GAG scaffolds for bone tissue engineering, J Mech Behav Biomed Mater 2 (2009) 202-9.

[15] R.L. Baldwin, How Hofmeister ion interactions affect protein stability, Biophysical Journal 71 (1996) 2056-2063.

[16] J.S. Pieper, A. Oosterhof, P.J. Dijkstra, J.H. Veerkamp, T.H. van Kuppevelt, Preparation and characterization of porous crosslinked collagenous matrices containing bioavailable chondroitin sulphate, Biomaterials 20 (1999) 847-858.

[17] S.V. Beekmans, D. Iannuzzi, Characterizing tissue stiffness at the tip of a rigid needle using an opto-mechanical force sensor, Biomed Microdevices 18 (2016) 15.

[18] L. Buttafoco, P. Engbers-Buijtenhuijs, A.A. Poot, P.J. Dijkstra, W.F. Daamen, T.H. van Kuppevelt, I. Vermes, J. Feijen, First steps towards tissue engineering of small-diameter blood vessels: preparation of flat scaffolds of collagen and elastin by means of freeze drying, J Biomed Mater Res B Appl Biomater 77 (2006) 357-68.

[19] L.E. Freed, G.C. Engelmayr, Jr., J.T. Borenstein, F.T. Moutos, F. Guilak, Advanced material strategies for tissue engineering scaffolds, Adv Mater 21 (2009) 3410-8.

[20] J.H. Wen, L.G. Vincent, A. Fuhrmann, Y.S. Choi, K.C. Hribar, H. Taylor-Weiner, S. Chen, A.J. Engler, Interplay of matrix stiffness and protein tethering in stem cell differentiation, Nat Mater 13 (2014) 979-987.

[21] A. Buxboim, I.L. Ivanovska, D.E. Discher, Matrix elasticity, cytoskeletal forces and physics of the nucleus: how deeply do cells 'feel' outside and in?, Journal of Cell Science 123 (2010) 297-308.

[22] A.J. Engler, S. Sen, H.L. Sweeney, D.E. Discher, Matrix Elasticity Directs Stem Cell Lineage Specification, Cell 126 (2006) 677-689.

[23] A.D. Covington, Tanning Chemistry: The Science of Leather, RSC Publishing2011.

[24] W.H. Michael Redwood, Tanning Chemistry, The Science of Leather, Royal Society Of Chemistry 2015.

[25] R. Parenteau-Bareil, R. Gauvin, F. Berthod, Collagen-Based Biomaterials for Tissue Engineering Applications, Materials 3 (2010) 1863.

[26] J.S. Pieper, P.B. van Wachem, M.J.A. van Luyn, L.A. Brouwer, T. Hafmans, J.H. Veerkamp, T.H. van Kuppevelt, Attachment of glycosaminoglycans to collagenous matrices modulates the tissue response in rats, Biomaterials 21 (2000) 1689-99.

[27] N.A. Hosper, A.J. Eggink, L.A. Roelofs, R.M. Wijnen, M.J. van Luyn, R.A. Bank, M.C. Harmsen, P.J. Geutjes, W.F. Daamen, T.H. van Kuppevelt, D.M. Tiemessen, E. Oosterwijk, J.J. Crevels, W.A. Blokx, F.K. Lotgering, P.P. van den Berg, W.F. Feitz, Intra-uterine tissue engineering of full-thickness skin defects in a fetal sheep model, Biomaterials 31 (2010) 3910-9.

[28] J.S. Park, J.S. Chu, A.D. Tsou, R. Diop, Z. Tang, A. Wang, S. Li, The Effect of Matrix Stiffness on the Differentiation of Mesenchymal Stem Cells in Response to TGF-β, Biomaterials 32 (2011) 3921-3930.

[29] W.J. Hadden, J.L. Young, A.W. Holle, M.L. McFetridge, D.Y. Kim, P. Wijesinghe, H. Taylor-Weiner, J.H. Wen, A.R. Lee, K. Bieback, B.N. Vo, D.D. Sampson, B.F. Kennedy, J.P. Spatz, A.J. Engler, Y.S. Choi, Stem cell migration and mechanotransduction on linear stiffness gradient hydrogels, Proc Natl Acad Sci U S A (2017).

[30] Y.M. Ju, B. Yu, T.J. Koob, Y. Moussy, F. Moussy, A novel porous collagen scaffold around an implantable biosensor for improving biocompatibility. I. In vitro/in vivo stability of the scaffold and in vitro sensitivity of the glucose sensor with scaffold, J Biomed Mater Res A 87 (2008) 136-46.

SUMMARY AND FUTURE PERSPECTIVES

Samenvatting en toekomstvisie

Summary

Tissue engineering, defined as the process of regenerating tissues or organs to restore or enhance normal function, is a discipline which has been extensively investigated over the last 25 years with the ultimate aim to reduce dependence on donor organs. Many tissue engineering strategies make use of constructs (scaffolds) to be implanted in a tissue defect to restore tissue function. Occasionally constructs remain in the body permanently to keep fulfilling their function. However, the scaffold mostly functions as a biodegradable artificial extracellular matrix which temporarily provides mechanical strength, carries pre-seeded (stem)cells or guides cells from the neighboring tissue into the defect and facilicates regeneration by the body's own healing mechanisms. During regeneration, infiltrating cells slowly degrade the implant and create their own ECM resulting in newly formed body tissue. The scaffold plays a key role in the regenerating process, therefore the choice of the biomaterial is of utmost importance. In general, scaffolds can be prepared from 1) decellularized tissues or 2) molecularly defined biomaterials from natural or synthetic origin. In **Chapter 1** an overview was given of molecularly defined biomaterials and processing methodologies used in the field of tissue engineering and their potential clinical applications.

Type I collagen has been extensively used as a biomaterial for the preparation of implants due to its wide availability from various animal sources, biodegradability and excellent cross-species biocompatibility. In addition, collagen implants can easily be tailored by straightforward casting and molding processes to fit the defect that has to be restored. Collagen implants can also be functionalized with specific biologically active components such as glycosaminoglycans or growth factors stimulating regeneration of the target tissue. However, less attention has been paid to the mechanical characteristics of collagen scaffolds. Ideally, scaffolds would display similar mechanical characteristics as the ECM of the target tissue. The aim of this thesis was to develop novel methods to modulate the (mechanical) properties of collagen constructs thereby expanding the possibilities for clinical applications.

In **Chapter 2**, a systematic review and meta-analysis was presented of all pre-clinical and clinical studies performed on tissue engineering of the urethra. The aim was to find evidence for the efficacy for the use of tissue engineering for urethral repair and investigate trends with regard to the use of cellular vs. acellular implants, the type of biomaterial used, and the animal model chosen. After critical evaluation of the data,

the efficacy of the use of tissue engineering for urethral repair could not be established due to a lack on randomized controlled studies. However, outcome data of the meta-analysis (complication-rate, functionality, and study completion) was comparable to standard treatments described in clinical literature indicating that tissue engineering is a promising strategy for urethra repair.

In **Chapter 3**, a novel method is described to induce elasticity in rigid tubular scaffolds consisting of solely type I collagen. In the extracellular matrix, collagen usually provides structural support and adds strength but not elasticity, a characteristic required in dynamic tissues such as lungs, blood vessels, the gastro-intestinal tract and the urogenital system. By straightforward chemical crosslinking in combination with compression and corrugation tubular collagen scaffolds with a folded or corrugated structure were created. This unique corrugated structure allowed the scaffold to be repeatedly stretched in longitudinal direction without damaging the scaffold itself. When the stretching force was released, the scaffold instantly returned to its corrugated state showing that the scaffold had shape memory. Other important parameters such as cytocompatibility and mechanical strength were not affected by this new processing method.

Most tubular structures in the human body however are not elastic in longitudinal, but in radial direction. In **Chapter 4** it was shown how the method do induce elasticity described in Chapter 3 was adjusted in order to endow collagen scaffolds with elasticity in radial direction. By compressing the scaffold into a star-shape followed by chemical crosslinking, the star-shaped lumen of the scaffold was able to repeatedly expand upon exposure to internal pressure and close after the pressure has dropped (shape memory). This expanding and closing mechanism is also seen in many tubular tissues such as the esophagus, ureter and urethra. Expansion and closure of the collagen scaffold did not affect cell attachment as shown by cell culture under dynamic conditions mimicking voiding dynamics observed in the urethra.

In **Chapter 5**, a potential clinical application for shape memory collagen scaffolds was reported. By inverting the shape memory mechanism from the star-shaped scaffold as shown in Chapter 4, a self-expandable vascular implant was created consisting of solely type I collagen. It may be implanted using only minimal invasive surgical techniques in a compressed and folded state and subsequently deploy within one minute after implantation upon exposure to water. Cell culture with vascular endothelial cells

confirmed cytocompatibilty. This novel biodegradable vascular implant may be useful for vascular repair and may be an alternative for currently applied permanent implants prepared from synthetic or metal materials.

For other tissues, such as cartilage or bone, the scaffold should be rigid and stiff instead of elastic. In **Chapter 6**, a method is presented that can be applied to enhance the stiffness and rigidity of collagen scaffolds. By treatment with concentrated divalent salt solutions such as calcium chloride, collagen fibrils partly denatured causing shrinkage of the entire scaffold with preservation of the microporous structure. This shrinkage increased the stiffness of the scaffold 10-fold without affecting biocompatibility as assessed by subcutaneous implantations in a rat model. Using this new method collagen scaffolds may also be used for regeneration of hard tissues such as bone and cartilage without the need for additional components (e.g. ceramics or synthetic polymers) that are currently often added to collagen scaffold to enhance mechanical properties.

To conclude, type I collagen is a widely applied biomaterial in the field of tissue engineering, but clinical translation is often hampered by mismatched mechanical characteristics. In this thesis, collagen scaffolds were manipulated using a variety of methods, *i.e.* compression, corrugation, crosslinking and salt treatment, in order to alter shape and mechanical properties. These novel tools may be useful to prepare tailored-made collagen scaffold for the repair of many different tissues or organs.

Future perspectives

The need for alternative treatment options for tissue or organ repair is obvious. The world-wide lack of donor organ and the donor-related problem with immune rejection have stimulated the rise of tissue engineering since the early 90's [1]. Today, over 25 years later, patients in need of a new organ are still mostly dependent on organ donors as *de-novo* creation of new organs in a laboratory is more complicated than expected by early researchers. Expectations that researchers would be able to generate fully functional organs that could replace damaged organs are therefore still not met. Nevertheless, due to our growing knowledge of fundamental molecular mechanisms of processes in the human body step-by-step progress towards the ultimate goal of creating functional organs on demand is being made.

Tissue regeneration is an interplay of complex processes such as cellular signaling, proliferation/differentiation of (stem) cells and extracellular matrix remodeling. For the field of tissue engineering it is of importance to gain more insight in the mechanisms involved in this interplay. On the level of stem cells major progress has been made such as the discovery of the induced pluripotent stem cells [2] and the easily accessible adipose derived stem cells [3]. With external stimuli, *e.g.* growth factors or enriched culture media, the differentiation of these stem cells can be directed into one specific cell type in tissue culture plates. However, since an organ or tissue consist of more than one cell type in a specific three dimensional structure, an artificial extracellular matrix, or scaffold, is an essential ingredient in guiding the development of cells into functional tissue. Novel techniques such as 3D printing of artificial ECMs [4] and hydrogels [5] can play an important role as tools to study the development of (stem) cells in a 3D-environment as they show superior resemblance to the *in vivo* situation. Besides culturing in a 3D-environment, it is advantageous to culture cells under dynamic conditions as this reduces the discrepancies between static culturing conditions *in vitro* and the dynamic environment cells experience *in vivo* [6]. Ultimately, this would lead to a better extrapolation from *in vitro* results to an *in vivo* setting.

On the level of the scaffold, it has been shown that the 3D shape of scaffolds is important as it contributes to special mechanical characteristics such as elasticity and shape memory (Chapters 3 and 4). In addition, shape (*e.g.* the harmonic-like structure) can also play a role in the development of cells. Caco-2 cells, a human epithelial cell line derived from a colon carcinoma, cultured on a type I collagen scaffold with a

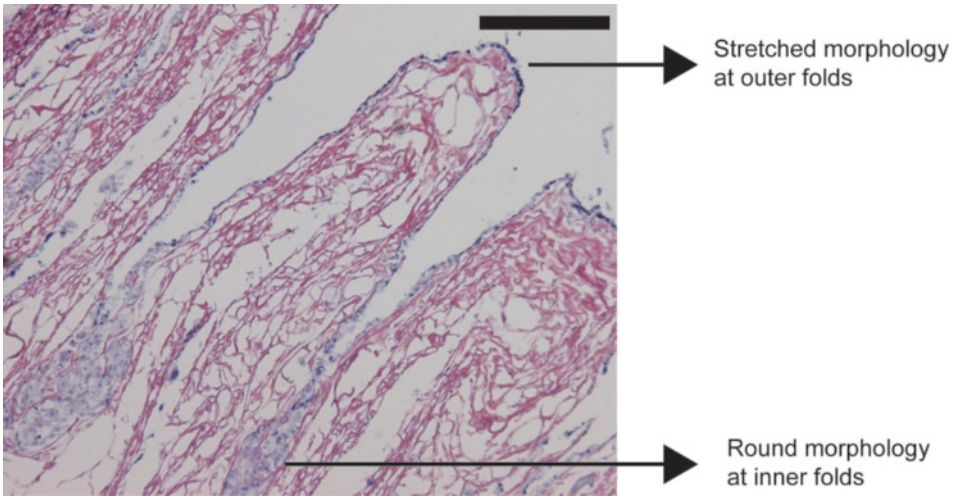


Figure 1. Caco-2 cells seeded on collagen scaffold with folded structure. The 3D-structure of a collagen scaffold influenced the morphology of the cells. In the inner folds the cells display a round morphology while at the outer folds the cells display a stretched morphology. Scale bar = 400 µm.

folded structure similar as presented in Chapter 3 showed to have an entirely different morphologies dependent on the location within the scaffold (Figure 1). In the inner fold of the scaffold, the cells displayed a round morphology, while on the outside of the tip region, the cells displayed a stretched morphology (Figure 1). This indicates that a specific 3D-structure may have an effect on the differentiation of cells. In this respect, collagen scaffolds with various shapes, prepared using the techniques described in this thesis, can serve as a model to study the development of stem cells in a 3D environment which may yield more valuable information that 2D cultures in plastic culture plates.

Besides its role as a supportive structure for the cells, the scaffold also serves as signaling agent. In recent years it has become clear that cellular behavior is not only guided by external stimuli but also by intrinsic mechanical signals from the ECM, also called mechanotransduction [7]. It has been shown in several studies that the mechanical properties of the substrate to which cells attach have major influence on differentiation of stem cells [8]. For example, stiff scaffolds tend to stimulate differentiation of mesenchymal stem cells towards smooth muscle cells or bone lineage, whereas soft scaffolds direct the cells towards adipogenic and chrondrogenic differentiation [9, 10]. Current studies investigating the effect of mechanosignaling mostly use synthetic matrices prepared from polyacrylamide. With the scaffolds developed in this thesis, it may be possible to use scaffolds prepared from natural

biomaterials for these studies, which better resemble the natural microenvironment of (stem) cells.

In this thesis, novel methods are described to create type I collagen scaffolds with a tissue-specific shape and mechanical properties that better resemble the mechanical properties of the extracellular matrix of the tissue to be regenerated. Nevertheless, there may still be room for improvements with the respect to the composition of the scaffolds as the scaffolds described in this thesis consists only of type I collagen. A native extracellular matrix consists of multiple components that give it their structure and cell-adhesive properties, including collagen, elastin, laminin, fibronectin, and heparan sulfate [11]. By adding additional components to a type I collagen scaffold, it will better mimic the native extracellular matrix, which may ultimately result in better tissue regeneration [12]. However, as each tissue has its own complex characteristic ECM it seems impossible to exactly mimic its composition and translate this into a multi-component scaffold. In addition, this will result in complex preparation processes resulting in expensive scaffolds. However, as it is quite clear that the ECM of some tissues contains a characteristic component, *e.g.* type II collagen for cartilage [13], calcium phosphate for bone [14] or type IV collagen for epithelial tissue [15], it may be advantageous to add them to the type I collagen-based scaffold. It will be key to find a good balance between the degree of complexity of the scaffold and the enhanced effect of the additional components.

Next to the hurdles to be taken in fundamental research as described above, there are other hurdles to take for the tissue engineering society at the level of translational research. In the field of fundamental research, a lot of progress has and still is being made. However, when considering the potential of tissue engineering and the number of clinical applications, it may be concluded that tissue engineering is underperforming [16]. To let patients benefit from fundamental discoveries on the short-term, the efficiency of clinical translation must be increased.

The process of developing novel therapies does not only take place in the laboratory. It is a multidisciplinary process involving many parties including medical doctors, pharmaceutical industry, regulatory bodies and researchers. In the current situation, the communication is often very poor. From the initial phase of development, all parties should intensively collaborate in order to maximize the chance of developing a successful therapy. As medical doctors are treating patients, they have the

knowledge about the clinical need and which specifications novel medical devices have to meet. Straightforward properties such as handability in the operating room can easily be defined by the end-users. Consequently, researchers can take into account these requirements already early in the developmental process. On the other hand researchers have the obligation to actively go into the clinic and present novel developments and techniques. Another very important party, which is often neglected in current clinical translation, is the industry. They possess specific knowledge that is often lacking at universities or hospitals, especially with regards to the feasibility of industrial production and accompanying legal requirements. A simple example is the sterilization of the scaffolds, which can have significant effects on the characteristics of a scaffold such as mechanical properties or immune responses *in vivo* compared to *in vitro* results [17]. An active scaffold loaded with fragile growth factors may lose its activity after sterilization. Another example is the use of raw materials and chemicals. Currently, in the *in vitro* and pre-clinical stages of development often raw materials and chemicals are used that are not allowed to be used in the clinical phase. In this phase, pharmaceutical grade materials and chemicals have to be used and this can result in significant changes in the end-product which will reduce the efficiency of clinical translation even further. If researchers already take industrial issues into account during the developmental process, clinical translation may increase. An additional party involved in the process of clinical translation are the regulatory bodies which determine legislation and audit adherence to the regulation. In April 2017, the new European Medical Device Regulation was released which is more defined and strict compared to previous regulation [18]. The introduction of the strict regulation was necessary to guarantee patient safety as medical devices became more complicated over the years. For researchers, it is difficult to apply every single rule of this regulation because indepth knowledge is lacking. Clinical translation would be stimulated when regulatory bodies would actively communicate with researchers and aid them to comply with the Medical Device Regulation. Another critical step in the translational chain are pre-clinical studies. As shown in Chapter 2, the quality of animal studies in the field of urethral tissue engineering is poor, especially with regard to experimental setup and reporting of results [19]. A randomized controlled experimental setup should be used to increase the reliability of study outcome. Only well-designed animal studies should be allowed, on one hand to increase efficiency of translation of tissue-engineered product to the clinical and on the other hand to extract as much valuable data from animals as possible from an ethical point of view. Furthermore we should strive for a system where all animal studies are centrally registered and where study

results are published open access analogous to a database such as ClinicalTrials.gov from the National Institute of Health (USA).

As the predictive value of animal studies has become a topic of discussion, research for alternatives gained interest. Tissue engineering can also play an important role in the development of these alternatives. Developments such as 3D cell cultures (*e.g.* organoids) [20] and organ-on-a-chip technologies [21, 22] are already used for screening of drugs *in vitro.* Collagen scaffolds may be used to create artificial organs for comparable applications. For example, a collagen scaffold resembling the morphology of intestines with the crypt and villus structure seeded with $lgr5^+$ stem cells similar as shown in figure 1, may form artificial intestinal tissues organs which can be used for research purposes.

In this thesis, new methods are described to modify collagen scaffold and endow them with desired properties for tissue specific applications opening up new opportunities for the field of collagen-based tissue engineering. On the one hand the techniques may be very useful to develop scaffolds which can be used as a platform to study the tissue regeneration *in vitro* in a setting better resembling the microenvironment that cells would experience *in vivo*. On the other hand, the techniques presented in this thesis can be valuable tools to create tissue-specific scaffolds resembling the native composition and the native morphological and mechanical properties. Consequently, it may be possible to use collagen-based scaffolds for the repair of a multitude of tissues. If researchers collaborate with clinicians, industry and regulatory bodies, and keep investing in fundamental research to elucidate the molecular pathways of tissue development and regeneration, artificial organs on demand could become reality in the future.

Samenvatting

Tissue engineering is een discipline in de medische wetenschappen die de laatste jaren sterk is op komen zetten als gevolg van het grote tekort aan donorweefsel. Tissue engineering heeft als doel om nieuwe weefsels of organen kunstmatig te creëren zodat ziek of beschadigd weefsel vervangen kan worden. Een veel toegepaste methode hiervoor is het gebruik van biologisch afbreekbare constructen, zogenaamde 'scaffolds', die worden geïmplanteerd op de plaats van het defect waar weefselvorming nodig is. Deze scaffold heeft als taak om cellen uit omliggende weefsels naar het defect te leiden en om tijdelijk stevigheid te bieden aan het gehele weefsel. De scaffold fungeert met andere woorden als een kunstmatige en tijdelijke extracellulaire matrix. Tijdens het regeneratieproces breken cellen de scaffold langzaam af en creëren ze hun eigen extracellulaire matrix. Na verloop van tijd zal de scaffold helemaal afgebroken zijn en zal het oorspronkelijke defect helemaal gevuld zijn met lichaamseigen weefsel. Omdat de scaffold een zeer belangrijke rol speelt in het regeneratieproces is de keuze voor het biomateriaal waarvan de scaffold gemaakt erg van belang. Er kan onderscheid gemaakt worden tussen twee klassen van materialen: 1) gedecellulariseerde weefsels en 2) moleculair gedefinieerde materialen van biologische of synthetische origine. In Hoofstuk 1 is een overzicht gegeven van moleculair gedefinieerde materialen die veel worden gebruikt inclusief fabricatiemethoden en mogelijke klinische toepassingen.

In **Hoofstuk 2** wordt een overzicht gegeven van alle preklinische en klinische studies over het gebruik van tissue engineering voor plasbuisreconstructie in de vorm van een systematisch review met meta-analyse. Het doel van dit review was om bewijs te vinden voor de doelmatigheid van het gebruik van tissue engineering voor het behandelen van plasbuisdefecten. Daarnaast is onderzocht of het toevoegen van (stam)cellen, de materiaalkeuze en het gekozen diermodel invloed hadden op de uiteindelijke uitkomst. Na een kritische evaluatie bleek dat er geen harde conclusie getrokken kan worden over de doelmatigheid van tissue engineering door het ontbreken van controle groepen in de studies. Uit de data van de meta-analysis kon wel geconcludeerd worden dat de mate van complicaties, functionaliteit en studieuitval vergelijkbaar was met conventionele behandelingen voor plasbuisreconstructie. De resultaten in dit hoofdstuk laten zien dat tissue engineering voor plasbuisreconstructie veelbelovend kan zijn mits nieuwe studies op een goede manier worden opgezet en uitgevoerd.

In de extracellulaire matrix geeft collageen over het algemeen stevigheid aan de weefselstructuur, maar het draagt niet bij aan de elasticiteit van het weefsel. Voor dynamische weefsels zoals het gastro-intestinaal of urogenitaal stelstel is dit echter essentieel. In **Hoofdstuk 3** is een nieuwe methode beschreven om buisvormige scaffolds bestaande uit type I collageen elastische eigenschappen te geven. Door gebruik te maken van een chemische koppelingsreactie in combinatie met compressie- en vouwstechnieken is een scaffold gemaakt met een geplooide structuur die herhaaldelijk kan worden opgerekt in de lengterichting zonder dat er schade aan de scaffold ontstaat. Na het strekken keert de scaffold uit zichzelf terug naar de geplooide toestand. Deze nieuwe methode geeft de collageenscaffold een vormgeheugen zonder de celbiocompatibiliteit en mechanische sterkte aan te tastes.

De meeste buisvorminge structuren in het menselijk lichaam zijn echter niet elastische in de lengte richting maar in de radiale richting (van binnen naar buiten). In **Hoofdstuk 4** wordt uitgelegd hoe de methode om elastische collageenscaffolds te maken kan worden aangepast om scaffolds te creëren die elastische eigenschappen bezitten in de radiale richting. Door de buisvormige scaffold samen te drukken tegen een stervormige staaf en vervolgens met een chemische behandeling te fixeren ontstaat een collageenscaffold met een stervormig lumen. Dit stervormig lumen kan herhaaldelijk openen en sluiten door middel van een stijgende en dalende druk net zoals de scaffold met vormgeheugen uit hoofdstuk 3. Dit open- en sluit mechanisme komt ook voor in verschillende buisvormige weefsels zoals de slokdarm, urineleider en plasbuis. Na een celkweekexperiment onder dynamische omstandigheden welke urineren nabootst kon worden geconcludeerd dat het openen van het stervormige lumen geen negatieve gevolgen heeft voor de celhechting.

In **Hoofdstuk 5** wordt een mogelijk klinische applicatie gepresenteerd waarbij gebruikt wordt gemaakt van het vorm-geheugen mechanism van de stervormige-scaffold zoals beschreven in hoofdstuk 4. Door het omkeren van het vorm-geheugen is een vasculair implantaat gemaakt van type I collageen dat uit zichzelf kan ontvouwen. Door de compressie- en vouwtechnieken kan het implantaat geplaatst worden gebruikmakend van minimaal invasieve chirurgische methodes. Na plaatsing zal de scaffold in contact komen met water (bloed) en binnen een minuut ontvouwen en zich vastzetten tegen de wand van het bloedvat. Een celkweekexperiment heeft aangetoond dat cellen goed op het implantaat kunnen hechten. Dit innovatieve biologisch afbreekbaar implantaat kan mogelijk worden gebruikt voor het herstel van beschadigde bloedvaten en is wellicht

een alternatief voor de huidige implantaten gemaakt uit synthetische materialen of metalen.

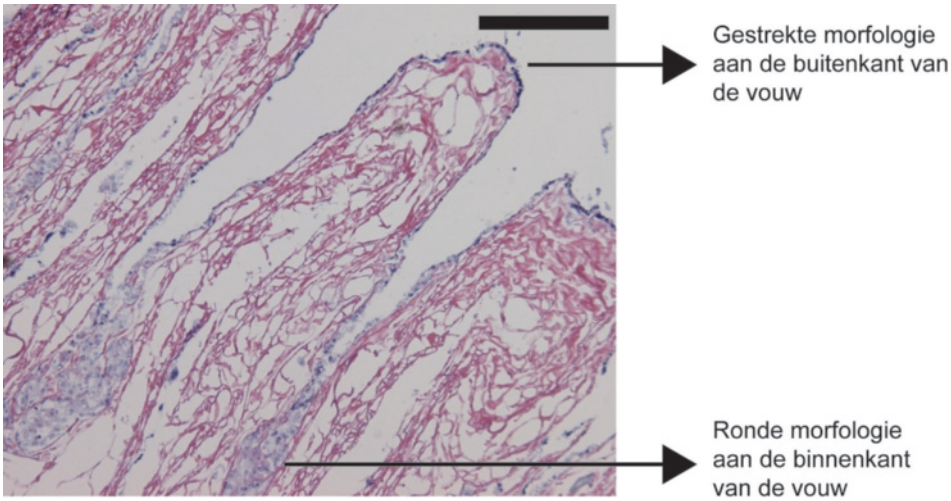
In andere weefsels zoals bijvoorbeeld bot biedt de extracellulaire matrix juist stijfheid en rigiditeit in plaats van elasticiteit. In **Hoofdstuk 6** is een nieuwe techniek gepresenteerd die gebruikt kan worden om collageenscaffolds stijver en meer rigide te maken. Door het collageen te behandelen met concentreerde zoutoplossingen zoals calcium chloride, worden de collageenfibrillen gedeeltelijk ontvouwen met als gevolg krimping van de collageenscaffold. Door deze krimping werd de stijfheid van de scaffold 10 maal zo hoog zonder dat de biocompatibliteit werd aangetast zoals aangetoond door middel van onderhuidse implantaties in een rat model. Deze nieuwe techniek biedt mogelijkheden om collageenscaffolds ook toepasbaar te maken voor de regeneratie van harde weefsels zoals bijvoorbeeld bot.

Samenvattend worden in dit proefschrift diverse innovatieve technieken beschreven zoals compressie, vouwing, chemische versteviging en zoutbehandelingen die gebruikt kunnen worden om de 3D structuur en mechanische eigenschappen van de scaffold te manipuleren. Met behulp van deze technieken kunnen op maat gemaakte scaffolds van type I collageen worden gecreëerd met de gewenste eigenschappen die nodig zijn voor optimale regeneratie van verschillende weefsels of organen.

Toekomstvisie

De noodzaak voor het ontwikkelen van alternatieve therapieën die het gebruik van orgaandonoren kunnen verminderen is duidelijk. Het wereldwijde tekort aan orgaandonoren en de bijkomende complicaties zoals afstotingsreacties hebben sinds het begin van de jaren negentig de ontwikkeling van tissue engineering in gang gezet [1]. Vandaag de dag, 25 jaar later, zijn patiënten nog steeds voor het grootste gedeelte afhankelijk van donororganen omdat het *de-novo* creëren van nieuwe organen in een laboratorium toch moeilijker is gebleken dan in eerste instantie voorspeld werd. Verwachtingen dat onderzoekers op korte termijn zouden slagen om functionele kunstorganen te maken die zieke organen kunnen vervangen zijn nog steeds een illusie. Desondanks, door de alsmaar groeiende kennis van fundamentele moleculaire mechanismen van processen in het menselijk lichaam, wordt er stap voor stap vooruitgang geboekt om het einddoel te bereiken; het maken van functionele organen op bestelling.

Weefselherstel is een complex samenspel van processen zoals cellulaire communicatie, celdeling, celdifferentiatie van stamcellen en modellering van de extracellulaire matrix (ECM). Voor tissue engineering is het van groot belang om meer inzicht te krijgen deze mechanismen. Op het gebied van stamcellen is enorme progressie gemaakt



Figuur 1. Caco-2 cellen gekweekt op een collageenscaffold met een gevouwen structuur. De 3D-structuur van een collageenscaffold heeft een invloed op de morfologie van de cellen. Aan de binnenkant van de vouw hebben de cellen een ronde morfologie terwijl de cellen aan de buitenkant een gestrekte morfologie hebben. Scale bar = 400 µm.

zoals de ontdekking van geïnduceerde pluripotente stamcellen [2] en de relatief eenvoudig verkrijgbare vetstamcellen [3]. Door externe signalen, zoals groeifactoren of kweekmedium, is het mogelijk om differentiatie van deze stamcellen te sturen richting een specifiek celtype in 2D in een kweekschaaltje op het laboratorium. Echter, een orgaan of weefsel bestaat uit meerdere celtypes met een specifieke 3D-structuur. Daarom is een kunstmatige ECM, ook wel scaffold genoemd, noodzakelijk om de celdifferentie en celgroei te sturen in de richting van functioneel weefsel. Nieuwe technieken zoals het 3D printen van ECM [4] en hydrogelen [5] kunnen een belangrijke rol spelen bij de bestudering van het gedrag van stamcellen in een 3D-omgeving omdat dit een betere weerspiegeling is van de situatie in het menselijk lichaam. Daarnaast kan het kweken onder dynamische condities, in bijvoorbeeld bioreactoren, de verschillen verkleinen tussen het kweken van cellen in het lab ten opzichte van hoe cellen groeien in ons lichaam [6]. Uiteindelijk kunnen deze methoden leiden tot een betere extrapolatie van een *in vitro* celkweekexperiment naar het menselijk lichaam.

Kijkend naar de scaffold is het duidelijk geworden dat de 3D-structuur zeer belangrijk is, omdat deze bijdraagt aan de mechanische eigenschappen zoals elasticiteit en vormgeheugen (hoofdstuk 3 en 4). Daarnaast kan de vorm (bijv. de harmonica-achtige structuur) van de scaffold ook een rol spelen in de differentiatie van cellen. Caco-2 cellen, een humane epitheel cellijn geïsoleerd uit een darmtumor, lieten na kweek op een collageenscaffold met een gevouwen structuur zoals weergegeven in hoofdstuk 3 een morfologie zien die afhankelijk was van de locatie in de scaffold (Figuur 1). Aan de binnenkant van de vouw hebben de cellen een ronde vorm, terwijl op de uiteinden van de vouwen de cellen een meer gestrekte morfologie hebben. Dit resultaat duidt er op dat de 3D-structuur van de scaffold een invloed heeft op celdifferentiatie. In dit opzicht zouden scaffolds met een specifieke structuur, gemaakt met de technieken beschreven in dit proefschrift, kunnen dienen als een model om het gedrag van stamcellen te bestuderen.

Naast een ondersteunende rol voor cellen om aan te hechten, heeft de scaffold ook een sturende functie. In de afgelopen jaren is het steeds meer duidelijk geworden dat het gedrag van cellen niet alleen bepaald wordt door externe stimuli, maar ook door de intrinsieke mechanische eigenschappen van de ECM, in dit geval de scaffold, ook wel mechanotransductie genoemd [7]. Diverse studies laten zien dat de mechanische eigenschappen van het substraat waarop de cellen gehecht zijn van grote invloed zijn op het differentiatiegedrag van stamcellen [8]. Scaffolds die erg stijf zijn, stimuleren

bijvoorbeeld de differentiatie van mesenchymale stamcellen richting spiercellen of bot. Minder stijve scaffolds zullen celdifferentiatie eerder in de adipogene of chrondrogene richting sturen [9,10]. Op dit moment worden in studies waarin mechanotransductie wordt bestudeerd met name gebruik gemaakt van 3D-scaffolds gemaakt van polyacrylamide. Met de technieken beschreven in dit proefschrift is het mogelijk om scaffolds met verschillende vormen en mechanische eigenschappen te maken van een natuurlijk biomateriaal, zoals collageen, wat meer overeenkomt de natuurlijke omgeving van stamcellen in het menselijk lichaam.

In dit proefschrift zijn enkele nieuwe technieken beschreven om collageen type I scaffolds te maken met een weefselspecifieke structuur en mechanische eigenschappen die beter overeenkomen met de ECM van het weefsel dat hersteld dient te worden. Desondanks is er nog veel ruimte voor verbeteringen, met name wat betreft de samenstelling van de scaffold. In dit proefschrift bestaan de beschreven scaffolds namelijk alleen uit collageen type I. De ECM in ons lichaam bestaat echter uit verschillende eiwitten en suikers, zoals andere typen collageen, elastine, laminine, fibronectine, en heparansulfaat [11]. Door het toevoegen van deze componenten aan de collageenscaffold kan de ECM van het te herstellen weefsel beter nagebootst worden, wat uiteindelijk kan resulteren in betere weefselregeneratie [12]. Omdat de ECM een zeer complex geheel is met een samenstelling die voor ieder weefsel weer anders is, zal het in de praktijk onmogelijk zijn om de ECM precies na te maken. Daarnaast zou het maken van een exacte kopie van de ECM een zeer kostbaar proces worden. Nochtans is het duidelijk dat de ECM van verschillende weefsels vaak een specifieke component bevat die een belangrijke rol speelt voor de uiteindelijke eigenschappen. De ECM van kraakbeen bevat bijvoorbeeld veel collageen type II [13], terwijl de ECM van botweefsel juist veel calciumfosfaat bevat [14]. Epitheelweefsel bevat op zijn beurt weer collageen type IV [15]. Het is waarschijnlijk gunstig voor het weefselherstel om deze belangrijke componenten toe te voegen aan de collageen type I scaffold. Voor onderzoekers is het zaak om een goede balans te vinden tussen de mate van complexiteit van de scaffold en extra voordelen van het toevoegen van extra componenten.

Naast de noodzaak om meer inzicht te krijgen in de fundamentele mechanismen van weefselherstel zijn er nog andere hordes die genomen moeten worden door de tissue engineering gemeenschap op het gebied van klinische translatie. Ondanks de grote voortgang die de afgelopen jaren geboekt is in het fundamentele onderzoek valt het aantal klinische toepassingen van tissue engineering in de praktijk nog tegen. Er kan

geconcludeerd worden dat tissue engineering ondermaats presteert vergeleken met de hoge verwachtingen van begin jaren 90 [16]. Om patiënten op korte termijn echt te laten profiteren van de nieuwe ontwikkelingen in tissue engineering zal de efficiëntie van de klinische translatie verbeterd moeten worden.

Het ontwikkelingsproces van nieuwe therapieën vindt niet alleen plaats in het laboratorium. Het is een multidisciplinair proces waarbij meerdere partijen betrokken zijn zoals clinici, de farmaceutische industrie, wet- en regelgevers en onderzoekers. Vandaag de dag is de communicatie tussen deze partijen vaak niet optimaal. Al vanaf de eerste fase van het ontwikkelingsproces zouden deze partijen intensief met elkaar moeten samenwerken om de kans dat een therapie succesvol toegepast kan worden bij patiënten te vergroten. Aangezien clinici dagelijks in aanraking komen met patiënten weten zij precies waar de problemen liggen met huidige therapieën en waar eventuele nieuwe therapieën aan moeten voldoen. Een simpele eigenschap van bijvoorbeeld een scaffold, zoals handelbaarheid op de operatiekamer, kan veel betere worden ingeschat door de eindgebruiker dan door de onderzoeker op het lab. Idealiter wordt deze klinische input al in een vroeg stadium in het ontwikkelingsproces meegenomen door onderzoekers. Van de andere kant bekeken hebben onderzoekers ook de verplichting om actief naar de clinici te gaan en hen te informeren over nieuwe mogelijkheden en technieken.

Een andere zeer belangrijke partij in het translatieproces, die er vaak niet bij betrokken wordt, is de farmaceutische industrie. Juist daar ligt enorm veel kennis die ontbreekt bij universiteiten en ziekenhuis vooral op het gebied van industriële productie en bijkomende wet- en regelgeving. Een simpel voorbeeld is bijvoorbeeld de sterilisatie van scaffolds. Sterilisatie kan namelijk een significant effect hebben op de scaffold, bijvoorbeeld op de mechanische eigenschappen, de immunogeniciteit, of de activiteit van toegevoegde groeifactoren [17]. Een ander voorbeeld waar onderzoekers vaak niet goed van op de hoogte zijn is regelgeving met betrekking tot het gebruik van grondstoffen en chemicaliën. Op dit moment worden bij *in vitro* en preklinisch onderzoek vaak materialen en chemicaliën gebruikt die niet toegestaan zullen zijn in klinisch onderzoek met patiënten. In deze fase van de ontwikkeling zijn namelijk grondstoffen en chemicaliën nodig die zijn goedgekeurd voor klinisch gebruik. Deze verandering kan soms enorme gevolgen hebben voor bijvoorbeeld de eigenschappen van de scaffold. Als wetenschappers zich hier al van bewust zijn in een vroeg stadium van het ontwikkelingsproces kunnen deze problemen voorkomen worden. De

efficiëntie van de translatie van laboratorium naar de patiënt zal hiermee waarschijnlijk verbeterd worden.

Verder hebben de organisaties die gaan over wet- en regelgeving een belangrijke rol in het ontwikkelingsproces van laboratorium naar de kliniek. Vanaf april 2017 is de er een nieuwe Europese regelgeving van kracht met betrekking tot medische hulpmiddelen, waaronder ook scaffolds vallen [18]. Deze nieuwe regelgeving is strenger en meer gedetailleerd dan de oude regelgeving die niet meer actueel was door de snelle ontwikkeling binnen tissue engineering in de laatste jaren. Voor onderzoekers is lastig om rekening te houden met al deze wetten en regelgeving omdat diepgaande kennis over dit onderwerp ontbreekt. Klinische translatie zou gestimuleerd kunnen worden als de wet- en regelgevers op een actieve manier communiceren richting de onderzoekers en hen helpen om te voldoen aan eisen van de medische hulpmiddelen regelgeving.

Een cruciale stap van om nieuwe therapieën bij de patiënt te krijgen is preklinisch onderzoek in diermodellen. Zoals is gebleken uit Hoofdstuk 2 van dit proefschrift, is de kwaliteit van preklinisch onderzoek, dit geval voor tissue engineering van de plasbuis, voor verbetering vatbaar. Voornamelijk de experimentele opzet en de rapportage van resultaten liet te wensen over [19]. Om de betrouwbaarheid van preklinisch onderzoek te verbeteren zijn gerandomiseerde studies met controlegroepen noodzakelijk. Om de efficiëntie van translatie van tissue engineering therapieën te vergroten, maar ook vanuit een ethisch oogpunt zouden in de toekomst alleen goed opgezette dierstudies uitgevoerd mogen worden. Daarnaast moet er gestreefd worden naar een systeem wat voor iedereen toegankelijk is waarin alle dierstudies wereldwijd geregistreerd staan en waarin resultaten gerapporteerd worden zoals dit ook al gebeurd voor klinische studies met patiënten met als voorbeeld ClinicalTrials.gov van het National Institute of Health (USA).

Naast ethische bezwaren is er ook steeds meer een discussie gaande over de voorspelbare waarde van dierstudies voor klinisch succes. Als gevolg hiervan heeft het onderzoek naar alternatieven voor dierexperimenten een vlucht genomen. Tissue engineering kan hierin een belangrijke rol spelen. Ontwikkelingen zoals 3D celkweken zoals organoids [20] en de orgaan-op-een-chip technologie [21,22] worden vandaag de dag zelfs al gebruikt voor het *in vitro* screenen van mogelijke nieuwe medicijnen. Collageenscaffolds kunnen wellicht ook gebruikt worden voor dit doel, bijvoorbeeld een scaffold die de vorm heeft van de darm (crypten en villi) kan worden bezaaid

met lgr5^{+} stamcellen vergelijkbaar met de situatie weergegeven in figuur 1. Op deze manier wordt een kunstmatig stukje darmweefsel gecreëerd die misschien gebruikt kan worden om de opname van medicijnen door de darm te bestuderen.

In dit proefschrift worden nieuwe methoden beschreven die gebruikt kunnen worden om collageenscaffolds een specifieke 3D structuur en andere mechanische eigenschappen te geven. Dit biedt nieuwe mogelijkheden voor het gebruik van collageenscaffolds in tissue engineering. Aan de ene kant kunnen de beschreven technieken gebruikt worden om scaffolds te maken die als platform kunnen dienen om het regeneratieproces beter *in vitro* te kunnen bestuderen. Aan de andere kant bieden de nieuwe technieken onderzoekers manieren om weefselspecifieke scaffolds te creëren die de ECM van het te herstellen weefsel beter kunnen nabootsen wat betreft 3D structuur en mechanische eigenschappen. Hopelijk kunnen op deze manier in de toekomst collageenscaffolds worden ingezet om de regeneratie van een variëteit aan organen en weefsel te stimuleren. Als onderzoekers beter gaan samenwerken met clinici, farmaceutische industrie, wet- en regelgevers, en blijven investeren in het ontrafelen van de fundamentele moleculaire mechanismes van weefselherstel dan zouden kunstmatige gemaakte organen op bestelling in de toekomst best eens realiteit kunnen worden.

References

[1] R. Langer, J.P. Vacanti, Tissue engineering, Science (New York, N.Y.) 260(5110) (1993) 920-6.

[2] K. Takahashi, S. Yamanaka, Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors, Cell 126(4) (2006) 663-76.

[3] U.D. Wankhade, M. Shen, R. Kolhe, S. Fulzele, Advances in Adipose-Derived Stem Cells Isolation, Characterization, and Application in Regenerative Tissue Engineering, Stem Cells International 2016 (2016) 3206807.

[4] F. Pati, J. Jang, D.-H. Ha, S. Won Kim, J.-W. Rhie, J.-H. Shim, D.-H. Kim, D.-W. Cho, Printing three-dimensional tissue analogues with decellularized extracellular matrix bioink, 5 (2014) 3935.

[5] Z. Wu, X. Su, Y. Xu, B. Kong, W. Sun, S. Mi, Bioprinting three-dimensional cell-laden tissue constructs with controllable degradation, 6 (2016) 24474.

[6] K. Uto, J.H. Tsui, C.A. DeForest, D.-H. Kim, Dynamically tunable cell culture platforms for tissue engineering and mechanobiology, Progress in Polymer Science 65 (2017) 53-82.

[7] T. Iskratsch, H. Wolfenson, M.P. Sheetz, Appreciating force and shape [mdash] the rise of mechanotransduction in cell biology, Nat Rev Mol Cell Biol 15(12) (2014) 825-833.

[8] J.H. Wen, L.G. Vincent, A. Fuhrmann, Y.S. Choi, K.C. Hribar, H. Taylor-Weiner, S. Chen, A.J. Engler, Interplay of matrix stiffness and protein tethering in stem cell differentiation, Nat Mater 13(10) (2014) 979-987.

[9] J.S. Park, J.S. Chu, A.D. Tsou, R. Diop, Z. Tang, A. Wang, S. Li, The Effect of Matrix Stiffness on the Differentiation of Mesenchymal Stem Cells in Response to TGF-β, Biomaterials 32(16) (2011) 3921-3930.

[10] G. Chen, C. Dong, L. Yang, Y. Lv, 3D Scaffolds with Different Stiffness but the Same Microstructure for Bone Tissue Engineering, ACS applied materials & interfaces 7(29) (2015) 15790-802.

[11] F.T. Bosman, I. Stamenkovic, Functional structure and composition of the extracellular matrix, The Journal of pathology 200(4) (2003) 423-8.

[12] R.J. Wade, J.A. Burdick, Engineering ECM signals into biomaterials, Materials Today 15(10) (2012) 454-459.

[13] M. Maldonado, J. Nam, The Role of Changes in Extracellular Matrix of Cartilage in the Presence of Inflammation on the Pathology of Osteoarthritis, BioMed Research International 2013 (2013) 284873.

[14] M. Tzaphlidou, Bone Architecture: Collagen Structure and Calcium/Phosphorus Maps, Journal of Biological Physics 34(1-2) (2008) 39-49.

[15] R. Kalluri, Basement membranes: structure, assembly and role in tumour angiogenesis, Nat Rev Cancer 3(6) (2003) 422-433.

[16] H. Kaul, Y. Ventikos, On the Genealogy of Tissue Engineering and Regenerative Medicine, Tissue Engineering. Part B, Reviews 21(2) (2015) 203-217.

[17] M. Sloff, Design of an artificial urostomy: the impact of sterilization on tissue remodeling, From urological tissue engineering to conduit innovations, Radboud University Nijmegen, Nijmegen, 2017.

[18] Regulation (EU) 2017/745 of the European Parliament and of the Council of 5 April 2017 on medical devices, amending Directive 2001/83/EC, Regulation (EC) No 178/2002 and Regulation (EC) No 1223/2009 and repealing Council Directives 90/385/EEC and 93/42/EEC in: E. Union (Ed.) 2017.

[19] L.R. Versteegden, P.K. de Jonge, J. IntHout, T.H. van Kuppevelt, E. Oosterwijk, W.F. Feitz, R.B. de Vries, W.F. Daamen, Tissue Engineering of the Urethra: A Systematic Review and Meta-analysis of Preclinical and Clinical Studies, European urology (2017).

[20] Y. Fang, R.M. Eglen, Three-Dimensional Cell Cultures in Drug Discovery and Development, SLAS discovery 22(5) (2017) 456-472.

[21] M.J. Wilmer, C.P. Ng, H.L. Lanz, P. Vulto, L. Suter-Dick, R. Masereeuw, Kidney-on-a-Chip Technology for Drug-Induced Nephrotoxicity Screening, Trends in Biotechnology 34(2) (2016) 156-170.

[22] S.N. Bhatia, D.E. Ingber, Microfluidic organs-on-chips, Nat Biotech 32(8) (2014) 760-772.

CURRICULUM VITAE
LIST OF PUBLICATIONS
PORTFOLIO
DANKWOORD

Curriculum Vitae

Luuk Versteegden (17 December 1988) was born in Lieshout and raised in Lierop (both North-Brabant, the Nederlands). In 2007 he graduated from the St. Willibrord Gymnasium in Deurne, after which he started with the bachelor study Molecular Life Sciences at the Radboud University in Nijmegen. During this bachelor, Luuk was a board member of the BetaBedrijvenBeurs Foundation in charge of organizing career events for students. After Luuk completed his bachelor study, he started with the master Molecular Life Sciences at the Radboud University Nijmegen. As part of this master, Luuk performed internships at the Department of Biochemistry and the Department of Urology, two research departments within the Radboud Institute for Molecular Life Sciences of the Radboud university medical center. In his first internship under supervision of Dr. Van Kuppevelt and Dr.ir. Daamen, Luuk investigated the use of microcapsules for local delivery of chemotherapeutic drugs. In his second internship under supervision of Dr. Oosterwijk, he investigated the use of tubular collagen constructs for urological applications such as urethra repair. In October 2012, Luuk obtained his master degree after which he started a PhD-project at the Department of Biochemistry under supervision of promoter Prof.dr. Brock and copromotores Dr. Van Kuppevelt and Dr.ir. Daamen to study the use of collagen constructs in regenerative medicine. The results of this research are described in this thesis and several international journals. Furthermore, Luuk has visited multiple international conferences and symposia where he presented his work. Luuk was member of the RIMLS PhD programme committee where he represented the PhD students of the Radboud Institute of Molecular Life Sciences. In addition he was responsible for the organization of the yearly PhD retreat. During the course of the PhD study, Luuk supervised several bachelor and master students from Biomedical Sciences and Medical Biology. After Luuk completed his PhD project in December 2016, he worked as a project manager at BioMed Elements in Nijmegen. As of January 2018, Luuk is working at Devro B.V. in Gendt as development project leader where he is applying his knowledge of collagen to develop collagen gels for the food industry.

Curriculum Vitae

Luuk Versteegden (17 december 1988) is geboren te Lieshout en getogen te Lierop (beiden Noord-Brabant). In 2007 behaalde hij zijn gymnasiumdiploma aan het St. Willibrord gymnasium te Deurne waarna hij startte met de bacheloropleiding Moleculaire Levenswetenschappen. Tijdens zijn bachelorstudie was Luuk bestuurslid van Stichting BetaBedrijvenBeurs waar hij betrokken was bij de organisatie van carrière-evenementen voor studenten. Na de bachelor te hebben afgerond, begon Luuk in 2010 met de aansluitende Engelstalige master Molecular Life Sciences. Tijdens de master liep Luuk stage bij de afdeling Biochemie en de afdeling Urologie, twee onderzoeksafdelingen binnen het Radboud Institute for Molecular Life Sciences verbonden aan het Radboudumc. Tijdens de eerste stage onder begeleiding van Dr. Van Kuppevelt en Dr.ir. Daamen deed hij onderzoek naar het gebruik van microcapsules voor locale afgifte van chemotherapie. In zijn tweede stage onder leiding van Dr. Oosterwijk onderzocht Luuk het gebruik van tubulaire collageenconstructen voor urologische toepassingen zoals de reconstructie van de urethra. In oktober 2012 behaalde Luuk zijn masterdiploma en hij startte aansluitend in november 2012 met zijn promotieonderzoek bij de afdeling Biochemie onder leiding van promotor Prof. dr. Brock en copromotores dr. Van Kuppevelt en Dr.ir. Daamen waarin het gebruik van collageen in de regeneratieve geneeskunde centraal stond. De resultaten van het onderzoek zijn beschreven in dit proefschrift en gepubliceerd in verschillende internationale tijdschriften. Daarnaast heeft Luuk meerdere internationale congressen en symposia bijgewoond waar hij zijn werk heeft gepresenteerd. Tijdens het promotieonderzoek was Luuk lid van het PhD-progammacomité waarbij hij de promovendi van het Radboud Institute for Molecular Life Sciences vertegenwoordigde en verantwoordelijk was voor de organisatie van de jaarlijkse PhD-retraite. Daarnaast heeft Luuk tijdens zijn promotieonderzoek verschillende bachelor- en masterstudenten van de studies Biomedische Wetenschappen en Medische Biologie begeleid. Na het afronden van zijn promotieonderzoek eind 2016 heeft Luuk gewerkt als projectmanager bij BioMed Elements te Nijmegen. Vanaf januari 2018 is Luuk werkzaam bij Devro B.V. als development project leader waar hij zijn opgedane kennis van collageen toepast bij de ontwikkeling van collageengelen voor de voedingsmiddelenindustrie.

List of publications

Versteegden LRM*, Ter Meer M*, Lomme RMLM, Van Der Vliet JA, Schultze Kool LJ, Van Kuppevelt TH, Daamen WF. "Self-expandable tubular collagen implants". 2018; *provisionally accepted*.

De Jonge PKJD, Sloff M, Janke HP, **Versteegden LRM,** Kortmann BBM, De Gier RPE, Geutjes PJ, Oosterwijk E, Feitz WF. "Ureteral reconstruction in goats using tissue-engineered templates and subcutaneous preimplantation". Tissue Engineering Part A. 2017 Dec 22. doi: 10.1089/ten.TEA.2017.0347.

Versteegden LRM*, De Jonge PKJD*, IntHout J, Van Kuppevelt TH, Oosterwijk E, Feitz WF, De Vries RBM, Daamen WF. "Tissue engineering of the urethra: a systematic review and meta-analysis of preclinical and clinical studies". European Urology. 2017 Oct;72(4):594-606. doi: 10.1016/j.eururo.2017.03.026.

Versteegden LRM, Van Kampen KA, Janke HP, Tiemessen DM, Hoogenkamp HR, Hafmans TG, Roozen EA, Lomme RMLM, Van Goor H, Oosterwijk E, Feitz WF, Van Kuppevelt TH, Daamen WF. "Tubular collagen scaffolds with radial elasticity for hollow organ regeneration". Acta Biomaterialia. 2017;52:1-8. Doi: 10.1016/j.actbio.2017.02.005.

Versteegden LRM*, Hoogenkamp HR*, Lomme RMLM, Van Goor H, Tiemessen DM, Geutjes PJ, Oosterwijk E, Feitz WF, Hafmans TG, Verdonschot N, Daamen WF, Van Kuppevelt TH. "Design of an elasticized collagen scaffold: a method to induce elasticity in a rigid protein". Acta Biomaterialia. 2016;44:277-85. Doi: 10.1016/j.actbio.2016.08.038.

Van Bracht E, **Versteegden LRM**, Stolle S, Verdurmen WP, Woestenenk R, Raavé R, Hafmans T, Oosterwijk E, Brock R, Van Kuppevelt TH, Daamen WF. "Enhanced cellular uptake of albumin-based lyophilisomes when functionalized with cell penetrating peptide TAT in HeLa cells". PLoS One. 2014;9(11):e110813. Doi: 10.1371/journal.pone.0110813.

Versteegden LRM, Sloff M, Hoogenkamp HR, Pot MW, Pang J, Hafmans TG, De Jong T, Smit TH, Leeuwenburgh SC, Oosterwijk E, Feitz WF, Daamen WF*, Van Kuppevelt TH*. "A salt-based method to adapt stiffness and biodegradability of porous collagen scaffolds". *In preparation*.

*contributed equally

Portfolio

Graduate School Radboud Institute for Molecular Life Sciences

Courses and Workshops	Year	ECTS
RIMLS Graduate School Introductory Course	2013	2.0
Hands-on-training in synthesis of evidence in animal sciences	2013	0.4
Course on laboratory animal science	2013	3.0
Scientific integrity course	2014	1.0
Academic writing	2014	3.0
Career management for PhD-students	2016	0.8
Seminars and Lectures		
Miscellaneous (including oral presentations)	2012-2016	4.0
Departmental seminars (including oral presentations)	2012-2016	4.0
Noon Spotlight (1x)	2013	0.1
Seminars (8x)	2013-2014	0.8
Technical Forum (6x)	2013-2014	0.6
Radboud Research Rounds (5x)	2014-2016	0.5
Symposia and Congresses		
RIMLS PhD-retreat, Wageningen, The Netherlands #	2013	0.5
World Conference on Regenerative Medicine, Leipzig, Germany #	2013	1.25
3rd IRB International PhD student symposium, Barcelona, Spain #	2013	1.0
Radboud Frontiers Symposium, Nijmegen, The Netherlands	2013	0.5
RIMLS PhD-retreat, Wageningen, The Netherlands #	2014	0.5
Matrix Biology Europe Conference, Rotterdam, The Netherlands #	2014	1.25
Radboud Frontiers Symposium, Nijmegen, The Netherlands #	2014	0.5
RIMLS PhD-retreat, Veldhoven, The Netherlands #	2015	0.75
TERMIS World Congress, Boston, United States ##	2015	1.5
Symposium in systemic review and meta-analysis in translational preclinical science, Aarhus, Denmark *	2015	0.75
RIMLS PhD-retreat, Veldhoven, The Netherlands *	2016	0.75
6th Freiberg Collagen Symposium, Freiberg, Germany *	2016	1.25
Teaching		
Lecturing Biomedical Sciences	2013-2014	1.0
Daily supervision of bachelor and master students of Medical Biology and Biomedical Sciences	2013-2016	13.6
Other		
- Member of RIMLS PhD Programme committee	2013-2014	1.0
- Organization of RIMLS PhD-retreat (2x)	2014-2015	4.0
		TOTAL: 50.27

Oral and poster presentations are indicated with a * and # after the name of the activity, respectively.

Dankwoord

Dit is ‘m dan, mijn proefschrift! Eindelijk klaar na vijfenhalf jaar! Het is hard werken geweest, en zeker de laatste loodjes waren af en toe best zwaar, maar ik ben stiekem best trots op het eindresultaat. Veel mensen hebben hieraan een steentje bijgedragen, want zonder hulp van (co)promotoren, collega’s, vrienden en familie is promoveren onmogelijk. Ik wil daarom graag iedereen die op wat voor manier dan ook heeft bijgedragen aan dit proefschrift enorm bedanken voor alle hulp en steun de afgelopen jaren.

Beste Toin en Willeke, ik was al bijna op weg Brussel om te starten met een master bedrijfskunde toen jullie me aanboden om promotieonderzoek te gaan doen bij de afdeling Matrixbiochemie. In eerste instantie was ik er zelf niet van overtuigd dat ik de ideale promovendus was. Maar jullie vertrouwen in mij gaf de doorslag om alsnog in Nijmegen te blijven. Tot op de dag van vandaag heb ik hier nog geen seconde spijt van gehad. Ik wil jullie beiden ontzettend bedanken voor jullie vertrouwen in mij.

Beste Toin, ik ken bijna niemand die zo bevlogen is in zijn vak als jij. Er is geen biochemische reactie in het lichaam waar jij de werking niet van kent. Jouw onuitputtelijk bron van kennis samen met je enthousiasme voor de biochemie hebben enorm veel bijgedragen aan dit mooie proefschrift wat hier nu voor ons ligt. Ik wil je ontzettend bedanken voor je begeleiding tijdens mijn promotietraject.

Beste Willeke, bedankt voor al je hulp de afgelopen jaren. Waar ik af en toe even van de planning afweek zorgde jij er altijd voor dat alles toch de juiste richting in ging en dat de deadlines gehaald werden. Jouw praktisch inzicht bij het opzetten van experimenten heeft me enorm geholpen, net zoals jouw scherpe blik op de manuscripten. Kleine foutjes die niemand zag werden door jou altijd opgemerkt. Bedankt voor alles!

Geachte professor Brock, beste Roland, ik wil u bedanken voor het feit dat ik mijn promotieonderzoek mocht uitvoeren bij de afdeling Biochemie en dat u mijn promotor bent geweest. De gesprekken met u over de vorderingen van mijn onderzoek hebben mij geholpen bij het stellen en realiseren van doelen met als gevolg dat ik mijn promotieonderzoek binnen een mooie termijn en met mooie publicaties heb kunnen voltooien.

Henk, mijn begeleider tijdens mijn masterstage, mede-promovendus, research partner, paranimf, maar vooral een hele goede vriend. Ik weet eigenlijk niet waar ik moet beginnen om jou te bedanken. Toen mijn masterstage naar York niet door kon gaan regelde jij dat ik via een constructie met de afdeling Urologie bij jou stage kon lopen. Deze stage heeft er uiteindelijk voor gezorgd dat ik promotieonderzoek kon gaan doen. In de eerste fase van mijn promotieonderzoek heb je me vaak laten zien hoe je op het lab experimentele opstellingen, mallen voor scaffolds, of andere zaken in elkaar knutselde. Dit geknutsel heeft er uiteindelijk toe geleid dat we samen enkele mooie artikelen hebben weten te publiceren. Naast het serieuze gedeelte hebben we ook heel veel gelachen en lol gemaakt, op het lab, maar zeker ook in de Laaf, in De Fuik, en tijdens de Vierdaagse. Het was echt een geweldige tijd! In onze toekomstige carrières ben ik ervan overtuigd dat onze paden nog vaak zullen kruisen. Henk, het is een eer om jou hier aan mijn zijde te hebben als paranimf. Ik wens jou, samen met Frederieke, Mia en Toby, het allerbeste voor de toekomst en ik hoop dat we elkaar nog lang blijven zien, niet alleen als collega's maar ook als vrienden. Henk, bedankt jonge!

Ook wil ik graag al mijn andere collega's van Matrixbiochemie bedanken! Arie, bedankt voor je hulp en de leuke gesprekken, van de Champions League tot wereldreizen, alles kwam voorbij. Ik hoop over een paar jaar nog een keer bij jou thuis op bezoek te komen in Bali. Els, bedankt voor de leuke tijd en voor je hulp, zeker bij al die honderden bestellingen die je hebt gedaan voor mij met bestelbriefjes die ik altijd maar voor de helft invulde (excuses nog daarvoor ☺). Elly, bedankt voor alles wat ik van je geleerd hebt op het gebied van collageen en tissue engineering. Zonder jouw ervaring was het een stuk moeilijker geweest om dit boekje vol te krijgen. Marianne, als U-genootjes hebben we veel lief en leed gedeeld. Verschillende hoogtepunten, maar ook dingen die een afgewezen artikel of een mislukt experiment relativeerde. Ik heb heel veel respect voor je. Bedankt Marianne! Theo, bedankt voor al je hulp op het gebied van microscopie en fotobewerking, maar zeker ook bedankt voor alle speciaalbiertjes die we samen in de Laaf gedronken hebben. Het was altijd kei gezellig!

Ik wil ook graag alle onderzoekers van Matrixbiochemie bedanken waar ik mee heb samengewerkt. De 'oude' garde, Etienne, Myrtille, Xander, Katrien, bedankt dat jullie mij wegwijs hebben gemaakt op de afdeling tijdens mijn stage en in het begin van mijn promotieonderzoek. Danique, bedankt voor de korte maar leuke samenwerking en heel veel succes gewenst met je verdere promotieonderzoek.

En dan de club van onderzoekers waar ik het grootste deel van mijn promotie samen mee heb gewerkt. Corien, Michiel, René en Sophieke, bedankt voor de super leuke tijd die ik met jullie heb gehad. Jullie zorgden ervoor dat ik altijd met plezier naar mijn werk ging, ook als het even tegenzat met experimenten of manuscripten. De vele gezellige avondjes in de Laaf en de AIO-weekendjes waren echt geweldig
Corien, het samen lekker klagen over mislukte experimenten of afgewezen artikelen deed me altijd erg goed, maar het lachen om onze flauwe grappen ga ik echt missen! Michiel, medebewoner van de Mansion, wat hebben we veel gelachen zowel op het werk als thuis. De trip naar Boston voor het TERMIS congres was echt een hoogtepunt, net als het jaarlijkse feest bij ons voor de deur tijdens de Vierdaagse! Geweldige herinneringen! René, de avondjes Laaf inclusief afterparty, de rondjes met de wielrenfiets, de whisky proeverijen, wat een mooie herinneringen uit onze promotietijd. Sophieke, jouw doorzettingsvermogen en '7 uur 's ochtends tot 2 uur 's nachts mentaliteit' hebben me vaak gemotiveerd tijdens mijn promotieonderzoek. Ook jouw vele culinaire restaurant tips kon ik altijd erg waarderen, ik heb vaak lekker gegeten dankzij jou!
We waren met z'n allen echt een geweldig clubje bij elkaar, zonder jullie zou mijn promotietijd nooit zo leuk zijn geweest. Super bedankt en ik hoop jullie allemaal nog lang te blijven zien.

I also want to thank all my students for their contribution to this thesis. Rens, Timo, Dimitris, Hemn, Ellen, Evy, Kenny, thank you all for the great work you did during your internships. I wish you the best in your future careers.

Ook wil ik alle collega's van de andere werkgroepen binnen de afdeling Biochemie hartelijk danken voor de hulp tijdens mijn promotieonderzoek, de leuke dagjes-uit en de gezellige jaarlijkse kerstdiners.

Naast Matrixbiochemie is er nog een afdeling waar ik me altijd erg thuis heb gevoeld, namelijk bij Urologie. Ik wil dan ook iedereen van de afdeling Urologie enorm bedanken voor de leuke samenwerking. Geachte prof. Feitz, beste Wout, u was dan wel niet officieel lid van mijn begeleidingsteam, maar zo voelde het altijd wel voor mij. Bedankt voor uw hulp en uw altijd motiverende woorden 'typen typen typen'. Egbert, Paul (Geutjes), bedankt voor jullie ondersteuning. Dorien, bedankt voor al je hulp op het lab met celkweekexperimenten en kleuringen, maar vooral bedankt voor je gezelligheid. Kees, bedankt voor je hulp, je interesse in mijn onderzoek en de leuke gesprekken. Marije, we hebben veel samengewerkt bij verschillende projecten, zoals het NovioTissue project, het gekrompen scaffolds project en het PhD-retreat committee. Bedankt voor de leuke

en gezellige samenwerking. Paul, bedankt voor de leuke tijd in de Mansion en de super leuke samenwerking op het lab. We hebben heel veel gelachen en lol gemaakt, maar wat hebben we samen veel geklaagd over de voortgang van ons systematic review. Vaak zaten we onderuit gezakt op het kennisplein met onze handen in het haar kijkend naar de meest onoverzichtelijke Excelsheet ooit gemaakt. Maar wat is het uiteindelijk een mooi artikel geworden! Bedankt Paul! Herr Peter, ik wil je graag bedanken voor de geweldige tijd in Nijmegen, zowel op het werk als thuis in de Mansion. Het was altijd super gezellig. Bedankt voor je hulp en interesse in mijn onderzoek, maar vooral bedankt voor je vriendschap!

Naast collega's van de afdeling Matrixbiochemie en Urologie zijn er nog veel andere collega's die hebben bijgedragen aan dit proefschrift.
Rob en Joanna, bedankt voor jullie hulp (en geduld) bij het schrijven van het systematic review. Het is uiteindelijk een prachtig artikel geworden in een heel mooi tijdschrift.

Tijdens mijn promotieonderzoek hebben we veel samengewerkt met het onderzoeksteam van de afdeling Heelkunde onder leiding van Prof. Harry Van Goor. Hartelijk dank voor de leuke en vruchtbare samenwerking. Roger en Edwin bedankt voor de hulp met de Zwick en de gezelligheid op jullie lab. Marja, bedankt voor onze leuke samenwerking die uiteindelijk heeft geresulteerd in een mooi hoofdstuk in dit proefschrift, wat hopelijk snel gepubliceerd gaat worden.

Huib, als ik de elektronenmicroscoop weer eens niet aan de praat kreeg, of ik als ik die ene opname net even niet scherp in beeld kreeg, dan hoefde ik maar te roepen of je kwam je me helpen. Bedankt!

Mark, bedankt voor de prettige samenwerking. Als we een technische vraag hadden of als er iets 3D-geprint moest worden dan stond je altijd direct voor ons klaar.

Patricia, in de afgelopen jaren hebben we regelmatig samengewerkt en dat was altijd erg leuk. Het was mooi om te zien hoe dicht het onderzoek in de medische industrie en de voedingsindustrie bij elkaar liggen. Onze samenwerking heeft dan ook zeker bijgedragen aan de resultaten beschreven in dit proefschrift. Hartelijk dank!

Geachte prof. Buma, beste Pieter, bedankt voor het feit dat u mijn mentor was tijdens mijn promotieonderzoek. Onze gesprekken en uw advies hebben me heel erg geholpen om op koers te blijven met mijn onderzoek.

I also want to thank all the members of the RIMLS PhD-programme committee for the great time I had together with you representing all PhD-students of the RIMLS. In addition, I want to thank the people of the organization committee of the yearly PhD-retreat for the great collaboration.

The research described in this thesis was part of the NovioTissue project, therefore I want to thank all the people involved in this project for the great and successful collaboration.

Kaspar, Pieter, Tonny, Bas, Jasper, Dion en Inge, bedankt voor de korte maar leuke tijd bij Future Chemistry/Biomed Elements. Ik vond het erg fijn dat ik bij jullie de mogelijkheid had om af en toe even naar de overkant van de straat te rennen om de laatste puntjes op de i te zetten voor dit proefschrift. Bedankt voor jullie hulp en interesse in mijn onderzoek.

I also want to thank all my Devro colleagues for their interest in my PhD-project and I appreciate the way I have been embraced by the Devro team during these first months. I look forward working together with all of you in the future.

Zonder collega's kun je niet promoveren, maar zonder familie en vrienden ook zeker niet! Roy, Rob, Luuk, Maarten, in onze studietijd hebben we veel lol gemaakt! Ook daarna toen we afgestudeerd waren hebben we veel ontzettend veel gelachen samen. Bedankt voor jullie vriendschap!
Peer, Lars, Willem, bedankt voor jullie interesse in mijn onderzoek en de leuke skivakanties.

Bert, Dirk, Dirk, Freek, Johnny, Karel, René, Rick, Robin en Thijs met jullie als vrienden was er in de weekenden altijd wat te beleven: voetbal kijken, een spelletje of gewoon wat ouwehoeren onder het genot van 'un lekker pilske'. Het was altijd heerlijk om na een weekje Nijmegen weer in Brabant te zijn. Bedankt mannen! Ook mijn vrienden uit Lierop, Michiel, Roel, de TOPpers van het zaalvoetbal, bedankt voor jullie interesse in mijn onderzoek en de leuke weekenden.

Marjo, Ron, Kris en Boris, de weekenden bij jullie op de Walstraat in Someren waren altijd kei gezellig! Bedankt!

Dianne, het grootste en vooral het zwaarste gedeelte van mijn promotieonderzoek stond jij aan mijn zijde. De leuke dingen die we samen deden zoals lekker uiteten, een terrasje pakken, met de backpack naar Thailand, of gewoon een rondje wandelen met Boris, waren altijd geweldig en zorgden ervoor dat ik iedere maandag weer helemaal relax en vol motivatie naar mijn werk ging. Jouw spontaniteit, pluk-de-dag mentaliteit en je motiverende woorden hebben me enorm geholpen tijdens mijn promotie, zeker als ik me weer eens te druk maakte over de voortgang van mijn onderzoek of om andere (vaak nutteloze) dingen. Bedankt voor alles wat je voor me gedaan hebt!

Pleun, mijn kleine zusje, die helemaal alleen naar New York kwam gevlogen nadat ik naar een congres in Boston was geweest! Wat was dat een gave vakantie! Het was echt super gaaf om met jou On Top of the Rock te staan en over de Brooklyn Bridge te lopen. Misschien dat dit jaar de rollen omgedraaid zijn en dat ik jou kan komen opzoeken in Amerika. Bedankt zusje!

Dirk, we hebben samen een hele tijd doorgebracht in Nijmegen. Onze gedeelde liefde voor pizza, shoarma, döner en kapsalon zorgde ervoor dat we vaak samen genoten van een heerlijk diners. Nu sta je hier als paranimf naast me, en waarschijnlijk sta jij over een paar jaar op mijn plek, aangezien ik al een tijdje niet meer in de enige Versteegden ben op Pubmed. Heel veel succes met je carrière, en hopelijk vieren we dan over een paar jaar weer een promotie! Dirk, bedankt!

Papa en mama, jullie hebben altijd alles voor mij gedaan en mij gesteund in alles wat ik deed. Jullie bleven interesse tonen in mijn promotieonderzoek, ook al was dit vaak niet mijn favoriete gespreksonderwerp. Papa, samen met jou in de weekenden een rondje maken op de wielrenfiets was geweldig, even ontspannen en bijbuurten, met als hoogtepunt onze fietsvakantie in Italië vorig jaar. Ik hoop dat we nog veel kilometers mogen maken samen. Mama, jij hebt er altijd voor gezorgd dat ik in Lierop een echt thuis had en dat ik niets te kort kwam. Voor jouw steun en zorgzaamheid ben ik echt heel dankbaar. Papa, mama, ik had me geen betere ouders kunnen wensen. Bedankt voor alles!

Luuk

Lierop, April 2018

Institute for Molecular Life Sciences

Radboudumc